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TUESDAY 27 MARCH 2001

0900 Comparative genomics and pathogenicity of mycoplasmas

S. RAZIN
Dept of Membrane and Ultrastructure Research, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel

Due to their minute genome and cell simplicity mycoplasmas were among the first organisms subjected to complete genome sequencing. The genomic projects have contributed most conspicuously to our understanding of the molecular biology and evolution of mycoplasmas. Accordingly, mycoplasmas evolved as a branch of gram-positive bacteria by reductive evolution, losing considerable portions of their ancestors' chromosomes in this process. The significant genome compaction was made possible by adopting a parasitic lifestyle. Supply of nutrients from their host facilitated the loss of genes for many assimilative, metabolic and regulatory processess. To keep to the parasitic lifestyle, mycoplasmas developed rather sophisticated mechanisms to colonize their host and resist the host's immune system. This required a significant number of genes devoted to adhesion and generation of antigenic variation systems, consisting mostly of membrane lipoproteins. Some of the lipoproteins and derived lipopeptides are active immunomodulators, playing an important role in mycoplasma pathogenesis.

The mycoplasmal genomic projects have brought us much closer to achieving the goal of complete deciphering, in molecular terms, of the machinery of a self-replicating cell. *M. genitalium* is, thus far, the organism closest to the theoretical minimal cell capable of self-replication. Global transposon mutagenesis was applied to test the effects of specific gene disruptions on growth. Of the 480 protein coding genes in *M. genitalium* 265 to 350 appear essential under laboratory growth conditions. In the case of DNA replication, transcription, and translation, it seems that the minimal set of genes has already been established in *M. genitalium* and *M. pneumoniae.* There is still very much to do to identify the unclassified open-reading frames (ORFs) that have no database match, prove experimentally the DNA-based predictions, and assign functions to proposed ORFs with hitherto unknown functions. Cell protein analysis, applying the 'Proteome' and 'Transcriptome' approaches are amongst the major directions currently taken to define structurally and functionally the entire complement of mycoplasmal cell proteins.

0945 Wall peptidoglycanless chlamydiae

JEAN-MARIE GHUYSEN and COLETTE GOFFIN
Centre for Protein Engineering, University of Liège, Belgium

Following internalization into host cells, chlamydial elementary bodies which are metabolically inert differentiate into reticulate bodies which undergo binary fission (and then differentiate back into elementary bodies which are released through cell lysis). Chlamydiae are peptidoglycanless gram-negative bacteria despite the fact that they have the information for the synthesis of lipid II which is the immediate precursor of peptidoglycan. Peptidoglycan assembly and remodelling rely on penicillin-binding acyl serine transferases that occur as free-standing penicillin-binding proteins (PBPs) and as modules of PBP fusions in which an acyl serine transferase module of class A or class B is fused, in a class-dependent manner, to distinct non-penicillin-binding modules having their own motifs of molecular recognition. PBP fusions of class A combine the required glycosyl transferase and acyl serine transferase activities through which the disaccharide peptide units borne by lipid II are converted into polymeric peptidoglycan. PBP fusions of class B possess an intra- and interprotein linker module that specifies which morphogenetic networks (involved in cell shape maintenance and septum formation) the associated acyl serine transferase module attaches to. *Chlamydia trachomatis* (Ctr) lacks the information for PBP fusions of class A. By denoting the PBPs by the numbering of the encoding ORFs, *C. trachomatis* has the information for a free-standing PBP Ctr D551 and two PBP fusions of class B, the 647 amino acid residues PBP Ctr D270 and 1080 amino acid residue PBP Ctr D682. The two PBP fusions of class B are inactivated by β-lactams at therapeutically achievable concentrations and their inactivation is associated with the formation of reticulate bodies of abnormal morphology, suggesting that these PBPs are involved, individually and collectively, in the synthesis, from lipid II, of an essential cell envelope polymer (whose structure remains unknown). The cell-cycle proteins FtsZ, a GTPase which holds other proteins of the divisome together and provides the driving force for cytokinesis, MraW, FtsW and RodA are strictly conserved among the peptidoglycan-containing bacteria. *C. trachomatis* produces MraW (ORF D272-encoded), FtsW (ORF D760-encoded) and RodA (ORF D726-encoded) but not FtsZ. The question of which connections may exist between the presumed wall polymer synthesized by the PBP fusions of class B and the process of cell division also remain unanswered.

Ref.: J.M. Ghuysen and C. Goffin. *Antimicrob. Agents Chemother*. 1999, 43 :2339-44 / C. Storey and I. Chopra. *Antimicrob. Agents Chemother*. 2000, 45 :303-5.

1145 Sex specific pathogenic Spiroplasmas of insects

MICHAEL E.N. MAJERUS
Dept of Genetics, University of Cambridge

Many species of invertebrate are infected by inherited bacterial symbionts. These symbionts have traditionally been classified into mutualists (beneficial), parasitic (harmful) and commensal (neutral). Symbionts that are predominantly transmitted vertically are almost totally dependent on the success of their host. It appears to follow that such symbionts should evolve strategies that increase host survival or at least ameliorate costs on their host. This thesis, however, ignores symbionts that manipulate their hosts to the symbionts' benefit, even when this is to the detriment of the host. The inheritance of these bacteria is typically through the female host. Consequently, a variety of strategies involving manipulation of host reproduction, usually in favour of female compared to male hosts have evolve. These include feminisation, parthenogenesis induction and male-killing.

Most research on these 'ultra-selfish' bacteria has concentrated on α-proteobacteria, such as *Wolbachia*. However, Spiroplasmas have been found to cause male-killing in hosts from three orders of insects, Coleoptera, Diptera and Lepidoptera. Typically male death occurs early in embryogenesis. The vertical transmission efficiencies of these Spiroplasmas is usually high (> 0.95). Prevalences range from < 0.01 to 0.8. The dynamics of these symbionts relies on daughters of infected females gaining some fitness advantage to compensate for the loss of the females' sons and any metabolic costs of bearing the bacterium. In the Coleoptera, factors contributing to fitness compensation have been identified. They include resource reallocation and reductions in sibling cannibalism of female offspring. In other orders, fitness compensation benefits to male-killing await discovery. The mechanism by which symbionts differentially kill male hosts is unknown, but appears to be density dependent.

The invasion of male-killing symbionts may have considerable consequences on host evolution, promoting sex role reversal and the evolution of suppresser genes, affecting

sex determination and mitochondrial DNA variability, and influencing traits such as clutch size. These will be discussed.

1145 A study of immunodominant membrane proteins associated with two related phytoplasmas

D.L. DAVIES, M.F. CLARK, A. MORTON and D.J. BARBARA
Horticulture Research International, East Malling, Kent ME19 6BJ

Phytoplasmas are a group of poorly understood wall-less prokaryotes associated with a wide range of plant diseases. They are not culturable and are difficult to purify. Infection in the plant is confined to the phloem tissue and phytoplasmas are spread by phloem-feeding insects such as leafhoppers. They can multiply in both plants and in insects but little is known about how they interact with either host. In this work we have studied the cell surface proteins found associated with phytoplasmas when serological detection techniques were developed as it is likely that these proteins may be involved in the interaction between the phytoplasmas and their hosts. The genes encoding immunodominant membrane proteins associated with aster yellows and clover phyllody phytoplasmas have been cloned and sequenced. There is extensive sequence similarity between the two proteins at the ends, including an identical leader sequence at the N-terminal that is cleaved to leave the mature protein, and a TM domain close to the C-terminus. The lowest similarity was found in the part of the molecule exposed on the cell surface. A comparison with the membrane proteins derived from five fruit tree phytoplasmas revealed several different formats with some molecules possessing TM domains at either the C terminus or the N-terminus or at both. Similarities between the different types reflected taxonomic relationships.

1400 Protoplast type L-forms - Changes in the cytoplasmic membrane after 30 years of life without a cell wall

JOHANNES GUMPERT and CHRISTIAN HOISCHEN
Institute of Molecular Biotechnology, PF.100813, D-07708 Jena, Germany

L-form cells are characterized by deficient or absent cell walls. Our protoplast type L-form strains of *Escherichia coli, Proteus mirabilis, Bacillus subtilis* and *Streptomyces hygroscopicus* have been cultivated for more than 1,000 generations in a cell wall-less state. They represent genetically stable variants showing extreme pleiotropic changes of their characters. Of special interest are the alterations in the cytoplasmic membrane. It represents the only cell envelope structure in L-form cells and has to fulfil the functions of the cytoplasmic membrane, of the cell wall, and of other surface structures.

A comparative analysis of the lipid components by 2D-TLC, GC, HPLC, and ESI-MS/CID-MS in cytoplasmic membranes of L-forms and their parent bacteria showed no substantial qualitative differences. However, significant quantitative changes, especially in phospholipid classes and molecular phospholipid species, were found. By using 2D-gelelectrophoresis, typical outer membrane proteins such as OmpA, OmpC, OmpF, and LamB were identified in the L-form membrane of strain *E. coli* LWF+. Although the L-form membrane is considered to be homologous to the inner membrane of the walled Gram negative bacteria, it obviously represents a hybrid structure, consisting of components from the inner and the outer membrane of the parent bacterium. L-forms of *P. mirabilis* and *E. coli* are suitable alternative cell systems for overexpression of recombinant proteins. In particular, they can be useful for the synthesis of membrane proteins and for a surface display of recombinant proteins. The combination of membrane-spanning sequences with a product gene allows the overexpression of soluble proteins in a membrane-bound and surface-displayed form.

1445 Animal mycoplasmas: dogma and disease

ROBIN NICHOLAS
Veterinary Laboratories Agency (Weybridge)

Mycoplasma mycoides subsp. *mycoides* small colony (SC) variant, the cause of contagious bovine pleuropneumonia (CBPP), is without doubt the most important of all the animal mycoplasmas. Furthermore, CBPP is the only bacterial disease recognised by the Office International des Epizooties as a list A disease because of the severe economic problems it causes. *M. mycoides* SC was the first mycoplasma to be isolated over 100 years ago although at the time it was thought to be a "filterable virus". It was a major cause of death and disease in cattle in Europe but was believed to have been eradicated by the early 20th Century. Infected European cattle, however, took the disease to Africa and Australia from where it eventually reached Asia. Today CBPP is the most serious cattle disease in sub-Saharan Africa while in Europe it appears sporadically but in a much less virulent form. Recent research has shown significant genetic and biochemical differences between isolates from Africa and Europe which may account for the lower mortality and morbidity rates seen in Europe but husbandry and other factors are also thought to play a part in the disease process. The source of the unexpected outbreaks of CBPP in Italy in the early 1990s, after 100 years of freedom, was never satisfactorily traced although some evidence suggested that it did not arise from Western Europe. Much remains to be known about this and other animal mycoplasmas including how they cause disease but recent research is helping to separate some of the facts from the fiction.

1600 L-form bacteria and their symbioses with plants

E.J. ALLAN
Dept of Agriculture & Forestry, University of Aberdeen, 581 King Street, Aberdeen AB24 5UA

L-form bacteria represent a type of growth where cell wall synthesis is suppressed while growth is sustained. L-forms can be derived from most bacterial types and generally appear as protoplast-like cells whose characters mainly differ from the parental forms through the modification, or complete lack, of their cell wall. Work, initiated at Aberdeen University by the late Professor Alan Paton indicated that L-form bacteria can form a symbiosis with plants with no adverse effects on plant growth and development. More recently research has shown that L-form bacteria can be successfully used as biological control agents giving protection against both bacterial and fungal pathogens. This talk will discuss the association between different L-form bacteria, mainly *Pseudomonas* and *Bacillus* spp., and plants. It will describe how the symbiosis has been confirmed using microscopy, ELISA, re-isolation and observation of genetically marked L-forms within plant tissue. Evidence for the induction of resistance by L-forms to the causative organism of grey mould, *Botrytis cinerea* will also be presented.

1615 Chlamydial vaccine development

DAVID LONGBOTTOM
Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ

Members of the family *Chlamydiaceae*are a diverse group of pathogens that cause a wide range of disease in both humans and animals, including STDs, infertility, blindness, respiratory disease, polyarthritis, conjunctivitis, metritis, enteritis, psittacosis and abortion. Early development of vaccines in the 1950s against the *C. psittaci* pathogen (now known as *Chlamydophila abortus*) responsible for abortion in sheep (Ovine enzootic abortion or OEA), which consisted of 'killed' whole organisms, was successful and led to the first commercially available chlamydial vaccine. In the 1960s and 1970s attempts to protect against trachoma, caused by the human pathogen *Chlamydia trachomatis*were largely unsuccessful. The 1980s saw the production of another successful vaccine against OEA, which was a temperature-sensitive attenuated live strain of *C. abortus*Following the identification of the major outer membrane protein (MOMP) and its ability to generate neutralising antibodies, the 1980s and 1990s has seen renewed attempts to produce MOMP-

based vaccines (membrane fractions, recombinant protein and peptides) to protect from chlamydial infection. However, these studies have produced disappointing results but did suggest that the confirmation of MOMP is a crucial factor for eliciting a protective immune response, and that antigens other than MOMP were required for sterile immunity. In addition, recent experiments in gene-knockout mice have demonstrated the key role that cell-mediated immunity, rather than humoral immunity, plays in protective immunity to intracellular organisms such as *Chlamydia*. Most recently, DNA vaccination has been shown to be effective in evoking a protective immune response to *Chlamydiaceae* spp. in various animal model systems. These findings coupled with the availability of whole chlamydial genomic sequences have demonstrated how DNA vaccination can be used as a tool to explore for new vaccine candidates.

POSTERS

CCS 01 Induction of Chitinases in Chinese cabbage plants after association with L-form bacteria

P.W.H.K.P. DAULAGALA[1,2], EUNICE J. ALLAN[1] and GRAHAM W. GOODAY[2]
[1]Dept of Agriculture & Forestry and [2]Dept of Molecular and Cell Biology, University of Aberdeen, Aberdeen AB25 2ZD

Plants respond to environmental stimuli and pathogen attack via induction of different defence mechanisms including the accumulation of pathogenesis-related proteins (PR proteins). In particular, studies have been focused on the antifungal activities of chitinases, because of the abundance of chitin in the cell walls of many potentially pathogenic fungi. Artificially induced symbioses of plants with L-form bacteria have previously been shown to have increased disease control. We have studied (i) the induction of chitinases in seedlings of Chinese cabbage (*Brassica campestris* var. *pekinensis*) after association with L-form *Pseudomonas syringae* pv. *phaseolicola* and (ii) the potential of these hydrolytic enzymes in controlling grey mould disease caused by *Botrytis cinerea*. Plant L-form associations were confirmed in Chinese cabbage by using a slide agglutination test, ELISA and re-isolation of the symbiont. Accumulation of chitinases was shown using fluorogenic 4-methylumbelliferyl substrates (for Family 18 chitinases) and dye-labelled substrates (for chitinases of Families 18 and 19). The development of grey mould in L-form associated plants was shown to be delayed as assessed by standard bioassays on detached leaves and on whole seedlings.

CCS 02 Distribution of CshA Fibrils amongst Mitis Group Oral Streptococci

D. ELLIOTT[1], M. MICHAEL[2], P.S. HANDLEY[1] and R. McNAB[2]
[1]Dept of Biological Sciences, University of Manchester, [2]Eastman Dental Institute, University College London

Many oral streptococci express large surface structures (fibrils and fimbriae) that have been implicated in adhesion of bacteria to oral surfaces. *Streptococcus gordonii* DL1 short peritrichous fibrils (61 nm long) are composed of cell-wall-anchored CshA adhesin (259 kDa). ELISA studies have previously shown that proteins immunologically related to CshA are expressed widely by other mitis group streptococci (including *S. mitis, S. sanguis,* and *S. oralis*) although it is not known whether the CshA-like proteins are structural components of fibrils in these organisms. To address this, we investigated the surface structures of several mitis group streptococci that express CshA. The identity of all strains was confirmed by 16s rDNA sequencing. Short fibrils (51-67 nm long) were present on all strains examined and fibril length was characteristic for each strain.

Immunoelectron microscopy was performed using antiserum specific for the N-terminal portion of *S. gordonii* DL1 CshA (N-CshA antiserum). For most strains, the gold label was located towards the tips of short fibrils, consistent with *S. gordonii* DL1 observations and with the molecular architecture of CshA. For *S. gordonii* MJ2, however, gold particles were significantly closer to the cell surface (25.5 ± 7.2 nm), corresponding to the mid-point of short fibrils (length 50.6 ± 9.4 nm). Additionally, two strains of *S. gordonii* did not react in ELISA with N-CshA antiserum but reacted strongly with antiserum to the C-terminal region of CshA. The results indicate that CshA may comprise short fibrils on a range of oral streptococcal species, although some strains may express non-conventional CshA molecules that nevertheless form fibril structures.

CCS 03 An investigation into L-forms derived from *Streptomyces Viridifaciens*

C.M.J. INNES and E.J. ALLAN
Dept of Agriculture & Forestry, University of Aberdeen, 581 King Street, Aberdeen AB24 5UA

This study aimed to investigate the induction and growth of L-forms derived from the filamentous actinomycete *Streptomyces viridifaciens*. L-forms were successfully induced in liquid medium using L-phase broth supplemented with lysozyme and penicillin. A stable culture, which did not require inducing agents in the growth medium was obtained which had a faster specific growth rate than the unstable L-form. The L-forms where spherical in shape and continued to increase in cell size and produce vacuoles after the exponential growth phase had finished. The L-forms were also osmotically fragile. The, presumably undifferentiated, cells however still had the capacity to produce pigments. In addition, bioassays using sensitive organisms indicated that the antibiotic, tetracycline was also produced. Thus, it is clear that these streptomycete L-forms have the ability to grow in a non-filamentous manner and produce secondary metabolites.

CCS 04 Differential regulation of HPr kinase in *Mycoplasma pneumoniae* and *Bacillus subtilis* – implications for carbon catabolite repression

KATRIN STEINHAUER[1], K.G. HANSON[1,2], WOLFGANG HILLEN[1] and JÖRG STÜLKE[1]
[1]Universität Erlangen-Nürnberg, Lehrstuhl für Mikrobiologie, Staudtstr. 5, 91058 Erlangen, Germany, [2]Birla Institute of Scientific Research, Statue Circle, Jaipur – 303 001, Rajasthan, India (kghanson@biologie.uni-erlangen.de)

Carbon catabolite repression (CCR) in bacteria is largely controlled by the components of the phosphotransferase system (PTS). In Gram positive bacteria, HPr kinase/phosphatase (HPr K/P) plays a central role in regulation of CCR. It carries out phosphorylation/dephosphorylation of Histidine containing protein (HPr) of PTS at Ser-46 finally resulting in regulation of catabolic operons. Since HPr K/P has also been found in Mycoplasmas, the smallest self-replicating organisms known, we are interested in studying its mode of regulation and role in CCR in *M. pneumoniae* and compare it with that from the more complex *B. subtilis*.

HPr K/P from *M. pneumoniae* and *B. subtilis* form hexamers and octamers, respectively. While HPr K/P of *M. pneumoniae* exhibits inherent kinase activity, that from *B. subtilis* exhibits phosphatase activity. This is supported by the finding that of *M. pneumoniae* HPr K/P has higher affinity for ATP compared with that of *B. subtilis.* In both cases fructose 1,6-bisphosphate (FBP) promotes kinase activity by counteracting the effect of P*i*. Thus, the functionally similar HPr K/P have different intrinsic activities in *M. pneumoniae* and *B. subtilis* which results in different regulation by low molecular weight effectors like FBP and P*i*. This difference in regulation probably reflects the different natural niches occupied by *B. subtilis* and *M. pnemoniae*, the former being primarily a soil bacterium while the latter is an intracellular parasite.

WEDNESDAY 28 MARCH 2001
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1000 The flow of antibiotic resistance genes in the environment

PETER M. HAWKEY
Division of Microbiology, School of Biochemistry and Molecular Biology & Antimicrobial Research Centre, University of Leeds, Leeds LS2 9JT

Antimicrobial agents have brought great benefits but antimicrobial resistance has been an inevitable consequence. The development of resistance is not surprising as most antibiotics are natural products produced by bacteria/fungi that inhabit the soil. There is evidence that resistance genes can be found in producer organisms and are transferred to pathogens (some aminoglycoside modifying enzymes and tetracycline resistance). Horizontal gene transfer (HGT) is the primary route whereby bacteria become resistant to antimicrobials. In the pre-antimicrobial era, bacteria carried self-transmissible plasmids lacking resistance genes. The movement of genes onto plasmids (the main vectors of HGT) can be via homologous recombination or mobilisation via insertion elements, transposons and the more recently recognised integrons. Approximately 50% of the antimicrobials used in the EU are for human medicine, the remainder in agriculture. Thus, a selective advantage is placed on environmental bacteria carrying resistance genes. In zoonotic species such as *Campylobacter* and *Salmonella* the influence on therapy of these "pre-evolved" strains is marked. Changes in medical practice have created patients who are infected by low grade pathogens, many coming from the environment. There is a need, therefore, to monitor these organisms and understand the molecular mechanisms for gene flow more fully.

1110 New resistance mechanisms in Gram positive pathogens

MARTIN GILL
Division of Immunity & Infection, Medical School, University of Birmingham, Birmingham

Efflux pumps are increasingly being recognised as important since, in combination with other resistance mechanisms, they may cause clinically significant resistance. These include the macrolide pumps MefA (*Streptococcus pyogenes*), MefE (*Streptococcus pneumoniae*) and MreA (*Streptococcus agalactiae*). Increased expression of chromosomal multi-drug pumps similar to NorA of *Staphylococcus aureus* have also been found, including PmrA of *Streptococcus pneumoniae*. Genome sequencing indicates that homologues to existing characterised pumps are found in a wide range of Gram-positives. Several pumps may exist in the same strain; up-regulated homologues of Bmr, Blt and EmrAB have been found in addition to NorA in a single strain of *S. aureus*. Drugs which are not affected by efflux-mediated resistance are being developed. Pump inhibitors are also being researched. Efflux pumps can contribute to intrinsic antibiotic resistance. Their inactivation in different species is being developed as a sensitive tool for screening for naturally occurring antimicrobial agents that would otherwise be effluxed from the cell. The repertoire of vancomycin resistance elements found in enterococci has been expanded and their diversity recognised. Most important is the isolation of vancomycin insensitive *S. aureus* (VISA) from clinical failures of vancomycin treatment. VISA may be difficult to detect in the clinical laboratory.

1150 Heteroresistance: resistant sub-populations in clinical isolates

HEINZ RINDER
Dept of Infectious Diseases and Tropical Medicine, University of Munich, Leopoldstr 5, D-80802 Munich, Germany

The simultaneous occurrence of drug-sensitive and -resistant subpopulations (heteroresistance) is a well-recognised phenomenon in staphylococci, especially *Staphylococcus aureus*. Heteroresistance has also been reported in *Gardnerella vaginalis* and *Cryptococcus neoformans*, but detection in non-staphylococcal organisms has been rare. In *Mycobacterium tuberculosis*, we encountered heteroresistance when we analysed problems during the validation of molecular resistance prediction tests. Drug resistance in *M. tuberculosis* is often linked to specific mutations in a limited number of resistance genes. Detection of these mutations and the ensuing prediction of phenotypic resistance was found to be reliable only if done on cultures. In contrast, validated culture-independent studies done directly with clinical samples are markedly absent in the literature although such an approach would result in the greatest time saving. To find out whether the resistance predicting genotypes of mycobacteria found after cultivation always give a good reflection of those in the original clinical sample we used a RFLP-based approach and cloning of amplicons from repeated PCR reactions as non-integrative methods to describe the composition of *katG*, *rpsL* and *embB* genotypes involved in resistance to isoniazid, streptomycin and ethambutol, respectively. The genotypes from sputum were then compared to the phenotypic resistance profiles after cultivation. A preliminary pilot study showed, by both genotypic methods, that mixed, heteroresistant populations could be detected in almost every fifth analysed sample (*katG*: 5 of 16; *rpsL*: 3 of 17; *embB*: 1 of 21). Direct sequencing, a widely used integrative method, repeatedly failed to detect heteroresistance. It is concluded that heteroresistance is a valid phenomenon in clinical tuberculosis. It is not rare and not restricted to a particular resistance gene, but it is obscured by cultivation as well as by some, not all, culture-independent resistance prediction tests.

1400 The ups and downs of antimicrobial resistance

MARC LIPSITCH
Harvard School of Public Health, Boston, USA

What factors determine the prevalence of resistance to antimicrobial agents (antibiotics and antiviral drugs) in populations of pathogens? How, and how fast, does this prevalence change in response to changes in antimicrobial use, infection control, and other interventions? What are the outstanding areas of uncertainty about these processes? This talk will discuss the use of population-biological models and experimental approaches to the problem of antibiotic resistance and its population dynamics. Particular focus will be on differences between community- and hospital-acquired pathogens.

1440 Study resistance with DNA arrays

KEN FORBES
Medical Microbiology, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD

The susceptibility of an organism to an antimicrobial is traditionally determined by the phenotypic test of whether its growth is inhibited by the drug. This strategy is constrained by the need to perform the test with a pure culture of the organism, the time that it takes for it to grow, and indeed, whether such a test is even feasible. Genotypic methods of resistance determination are intrinsically unaffected by phenotypic constraints as it is the genetic basis of the resistance that is determined. However, the genetic basis of

the resistance must be known and where this is not the case or where only a proportion of the resistance mechanisms are defined at the DNA level then genotypic methods may not offer as comprehensive a test as phenotypic susceptibility testing. Resistance can originate from exogenous gene(s) which confer resistance, specific mutations in genes endogenous to the cell, and in some cases a combination of these. Hybridisation of DNA from the test organism to specific probe sequences allows the detection of these characters, with the presence/ absence of a DNA sequence being technically simpler than the determination of point mutations in the target DNA. The former test can use long DNA probes, often derived from PCR products, while the latter requires the use of much shorter oligonucleotide probes and more complex hybridisation and detection systems. By performing a large number of hybridisation tests simultaneously on an array of DNA probes, and using standardised conditions, it is potentially possible to detect a multiplicity of resistance determinants. Multi-drug resistant, MDR, *Mycobacterium tuberculosis* is proving to be an attractive model system with which to develop array technology for the detection of antimicrobial resistance as arrays offer the potential to significantly speed up and improve on the current phenotypic tests.

1540 Molecular epidemiology of antimicrobial resistance: methods for clinical laboratories

ALEX van BELKUM
Erasmus University Medical Center Rotterdam EMCR, Dept of Medical Microbiology & Infectious Diseases, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands

Antibiotic resistant microorganisms can be traced by simply monitoring the dissemination of the resistance trait. Translated into a genetic approach, this implies that the resistance genes need to be unequivocally identified, whereas nucleic acid-based characteristics obtained for the genome of the pathogens involved can be instrumental in elucidating routes of spreading as well. During the past years a wide variety of methods suited for the genetic identification of bacterial species and their associated phenotypes have been developed. The usefulness of these methods will be illustrated for two prime species of clinically relevant types of resistant bacteria: methicillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci (VRE).

MRSA and VRA are both gram-positive microorganisms that can be grown on artificial media quite easily. Resistant isolates can be specifically selected for by inclusion of the appropriate antibiotics in the media. Once identified on the basis of the resistance profile, genetic characterisation can be performed. For this latter analysis our laboratory has been using various DNA mediated procedures over the past decade. Some of these methods aim at the characterisation of genomic DNA molecules. These latter methods, identified by acronyms such as REA, RFLP, PFGE, RAPD, AP PCR, MLST and others, will be shortly introduced and quality criteria and judgements will be discussed. Since typing systems should initially be validated on the basis of criteria such as typeability, resolving power and reproducibility, some of our studies addressing these criteria will be shortly presented in addition. However, prior to successful application in the clinical laboratory several other system characteristics need to be assessed. These include technical complexity, need for specialised reagents and personnel, availability and, last but not least, the costs. The various whole genome typing systems most frequently used for the genetic characterisation of VRE and MRSA will be positioned with respect to these criteria. Finally, some of the epidemiological mechanisms for MRSA and VRE will be discussed.

For both of the bacterial types described above, the resistance genes are part of large genetic elements. Both of these elements can be identified with the help of straightforward molecular diagnostics. PCR tests have been designed for the detection of the essential resistance-associated genes. Interestingly, the genetic elements, and the genomic environment in which they are residing, can be analysed for polymorphism, which in turn can be translated into strain-specific diagnostic and, consequently, epidemiological means. This enables not only the tracking of bacterial strains, but also the follow-up of the dissemination of a genetic element. For the transposon that gives rise to vancomycin resistance, for instance, we have been using DNA sequencing and hybridisation studies to document outbreaks of disease, due to dissemination of the transposon instead of patient-to-patient dissemination of a bacterial clone.

In conclusion, for both MRSA and VRE various excellent systems for whole-genome characterisation and for the identification of the genetic element associated with the resistance phenotype have become available over the past years. However, continuous refinement of the methodology is an ongoing process and modifications of established methods or the introduction of new methods (such as DNA chip technology) are still envisaged for the years to come.

1620 Expression of the *rdxA* gene in metronidazole-resistant and -sensitive *Helicobacter pylori*

STEPHANIE LATHAM[1], AGNÈS LABIGNE[2] and PETER J. JENKS[1]
[1]Dept of Medical Microbiology, Royal Free Hospital and UCL Medical School, Rowland Hill Street, London, [2]Institut Pasteur, rue du Dr Roux, Paris, France - Tel: 020 7794 0500 (ext. 4111); Fax: 020 7794 0433; email: pjenks2@hotmail.com

Objectives: Metronidazole resistance in *Helicobacter pylori* is highly associated with mutational inactivation of the *rdxA* gene. The aim of this study was to determine the expression of the *rdxA* gene in metronidazole-resistant and -sensitive *H. pylori* strains.
Methods: The complete *rdxA* gene was amplified by PCR and cloned into an expression vector. After overexpression, the recombinant protein was purified by affinity chromatography and used to raise polyclonal rabbit antiserum. Immunoblot analysis was performed on whole extracts of 15 metronidazole-sensitive and 60 metronidazole-resistant *H. pylori* strains isolated from the stomachs of mice treated with metronidazole. The nucleotide sequence of the *rdxA* gene of a number of metronidazole-resistant isolates was determined.
Results: All 15 metronidazole-sensitive *H. pylori* strains expressed the RdxA protein (MW of 24 kDa). In contrast, the RdxA protein was not expressed in 55 of the 60 metronidazole-resistant strains. The nucleotide sequence of the *rdxA* gene was determined for 11 of these strains: 9 strains contained frameshift (n = 8) or substitution (n = 1) mutations within the coding region of the gene, while in 2 strains the sequence was unchanged. RNA slot blots of these 2 strains demonstrated absence of specific mRNA production, implying mutation in the promoter region of the *rdxA* gene. In 5 of the 60 metronidazole-resistant strains, the RdxA protein was expressed. In each of these strains, the *rdxA* gene contained a substitution mutation within the coding region of the gene.
Conclusions: Expression of the *rdxA* gene is associated with susceptibility to metronidazole in *H. pylori*. The majority of resistant strains contained frameshift mutations within the *rdxA* gene with corresponding loss of expression of the RdxA protein. A small proportion of resistant isolates contained substitution mutations within the *rdxA* gene and expressed a non-functional protein of the same molecular weight as sensitive strains. Further work will determine whether this approach will be useful in discriminating between resistant and sensitive clinical isolates of *H. pylori*.

1640 Variation of the enzyme dihydropterin pyrophosphokinase and interactions with dihydropteroate synthase

YVONNE QVARNSTRÖM, MARIA JÖNSSON and GÖTE SWEDBERG
Dept of Pharmaceutical Biosciences, Div. of Microbiology, Biomedical Centre, Uppsala University, Uppsala, Sweden

The enzyme DHPS (dihydropteroate synthase) is well recognized as the target for sulphonamides. The enzyme preceding DHPS in the folate pathway, PPPK (dihydropterin pyrophosphokinase), is another interesting candidate drug target. We have cloned and sequenced the gene coding for PPPK *(folK)* from different clinical isolates of *Neisseria meningitidis* and *Streptococcus pyogenes*. Comparisons revealed some isolates with a mosaic structure in its *folK* gene, suggesting that horizontal transfer of genetic material has occurred. In *S. pyogenes* isolates with variation in PPPK also had a two amino acid insertion in DHPS associated with sulphonamide resistance. The PPPK enzymes from different isolates have been purified and characterised with respect to their kinetic properties.

The metabolic role of PPPK is to provide one of the substrates for DHPS. Earlier studies of DHPS enzymes have suggested that PPPK and DHPS enzymes need to have physical contact with each other for full enzyme activity. Substances that interfere with such interactions could lead to impaired growth and thus be used as inhibitory drugs. Studies of potential interactions between the enzymes have been initiated. The presence of the PPPK gene was shown to increase the growth rate of an *E.coli* mutant lacking DHPS in comparison with using DHPS alone for complementation.

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1400 Detection of antimicrobial resistance in clinical bacterial isolates by using molecular techniques

F.-J. SCHMITZ
Henrich-Heine University, Düsseldorf, Germany

The broad range of current PCR and DNA chip applications in clinical microbiology include the detection of pathogens, and the analysis of genomic alterations such as sequence and copy number alterations in bacterial genes and single nucleotide polymorphisms. The focus of this presentation should be the possible application of modern molecular methods for the detection of bacterial resistance genes and mechanisms.

As many of the genetic mechanisms of antimicrobial resistance have been become better understood, new molecular methods have proved to be useful for the confirmation of antimicrobial resistance in laboratory isolates and for the direct detection of such resistance in clinical specimens. Conventional culture and susceptibility test procedures for most pathogenic bacteria generally take 48-72 hours. The performance of these tests may be erratic because factors such as inoculum size or variability in culture conditions may affect phenotypic expression of resistance.

Testing is not only required for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community. However, the presence of a resistance gene not necessarily leads to treatment failure, because the level of expression may be to low. For example ß-lactamase production among Enterobacteriaceae is common, but the development of resistance is dependent on their mode and amount of expression.

The application of nucleic acid based technology is particularly useful for slow-growing or nonculturable microorganisms, the detection of point-mutations or certain genotypes.

1440 Erythromycin resistant *Streptococcus pneumoniae*

M.R. AMEZAGA, P.E. CARTER, P. CASH and H. MCKENZIE
University of Aberdeen, Dept of Medical Microbiology, Medical School Buildings, Foresterhill, Aberdeen AB25 2ZD

M phenotype resistance to erythromycin is mediated by active efflux and requires the *mef*(A) gene. The *mef*(A) sequences in *S. pneumoniae* and *Streptococcus pyogenes* are different. We previously demonstrated that an invasive, M phenotype, serotype 14 clone of *S. pneumoniae* expressed high levels of a variant of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a glycolytic enzyme that could be involved in energy generation for erythromycin efflux. However, other functions demonstrated in *S. pyogenes* such as plasmin binding and ADP-ribosylation may associate GAPDH with virulence. The aim of this study was to compare *gap* and *mef*(A) sequences in clinical isolates of *S. pneumoniae*. We have sequenced *gap* in 10 isolates of the M phenotype (serotype 14) and 13 other isolates. Sequencing of *gap* showed that the M phenotype serotype 14 clone has a base change that leads to the substitution of aspartate 137 for asparagine. Six M phenotype isolates of serotypes 9, 19 and 23 did not show the *gap* mutation nor the GAPDH variant. M phenotype isolates of serotype 14 had higher erythromycin MICs than those of other serotypes and sequencing revealed that they carried the *mef*(A)sequence of *S. pyogenes*, whereas isolates of other serotypes carried the *S. pneumoniae* sequence. Thus the invasive, M phenotype, serotype 14 clone of *S. pneumoniae* has several unusual features which merit further investigation.

1500 Identification of novel tetracycline resistance determinants in human commensal bacteria

CLAIRE M. MELVILLE, KAREN P. SCOTT, DERRY K. MERCER and HARRY J. FLINT
Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB

Bacterial resistance to antibiotics is now a global problem among groups of common pathogenic bacteria. Commensal gut bacteria whilst not a threat to human health are an important reservoir of antibiotic resistance genes with the potential for conjugal transfer to other bacteria, including pathogens.

A previously undescribed tetracycline resistance (Tc^R) gene, *tet*(W), was isolated by our group from the rumen anaerobe, *Butyrivibrio fibrisolvens* 1.230. This new ribosome protection type gene is carried on a 50 Kb mobile chromosomal element, Tn*B1230*, in the *B. fibrisolvens* strain 1.230. Sequence analysis of part of this transposon identified several open reading frames of interest. A gene > 99% homologous to *tet*(W) was also isolated from the human colonic bacteria, *F. prausnitzii* K10 and *B. longum* F8.

Tetracycline resistance (Tc^R) could be transferred from *Fusobacterium prausnitzii* K10 to the rumen anaerobe *B. fibrisolvens* 2221^R in an anaerobic filter mating, however *tet*(W) was not detected in the transconjugants. Instead a second novel Tc^R gene designated *tet*(32) was shown to be responsible for transmissible resistance in *F. prausnitzii* K10. This gene has also been characterised and sequenced.

The environmental distribution of these new genes, and in particular their contribution to previously unidentified tetracycline resistance in pathogenic bacteria is now highly significant.

1540 Antibiotic insusceptibility in eukaryotes: studies with *S. cerevisiae*

SIMON V. AVERY
School of Life and Environmental Sciences, University of Nottingham, University Park, Nottingham NG7 2RD

Eukaryotes, including yeasts, are considered insusceptible to tetracycline antibiotics. We found that deletion of *SOD1* (encoding Cu/Zn superoxide dismutase) rendered the eukaryotic model *Saccharomyces cerevisiae* highly susceptible to oxytetracycline (OTC): a *sod1*Δ mutant exhibited a >95% reduction in colony forming ability at 20 μg ml^{-1} OTC, whereas concentrations of up to 1000 μg ml^{-1} had no effect on the growth of wild-type *S. cerevisiae*. OTC insusceptibility was restored in the *sod1*Δ mutant by complementation with wild type *SOD1*. The effect of OTC was cytotoxic. OTC uptake was similar in wild type and *sod1*Δ cells. However, lipid peroxidation and protein oxidation were both enhanced during OTC exposure of the *sod1*Δ mutant but not the wild type. Several other tetracyclines and *S. cerevisiae* strains were tested and, of these, susceptibility was

also evident with doxycycline and in mutants defective in the Mac1p transcription factor. We propose that certain antioxidant proteins of *S. cerevisiae* protect against a novel mode of action of tetracyclines that is dependent on oxidative damage.

1600 Development of molecular typing markers for surveillance of antibiotic resistant *Helicobacter pylori* infecting British dyspeptics

N.C. ELVISS, R.J. OWEN and J. XERRY
Laboratory of Enteric Pathogens, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

Dyspepsia is a common symptom of *H. pylori* infection and its management has major cost implications in primary care. Eradication failure in 10% of patients due to antibiotic resistance is of growing concern. Our aim, for surveillance purposes, was to determine associations between clarithromycin (Cla)/metronidazole (Mtz) resistance *in vitro*, and strain genotype.

41 isolates of *H. pylori* with different Cla and Mtz susceptibilities, determined by disc diffusion and E-tests, were examined. These originated from gastric biopsies of patients with various gastrointestinal symptoms. PCR-based assays were used to determine presence of: *cag*A, *vac*A mid-region allelic forms (m1, m2), porin genes *hop*A, B, C and E, and Cla-associated mutations within the 23S rRNA gene.

H. pylori with dual (Mtz/Cla) resistance (10 isolates) were equally diverse as strains that were fully sensitive (11 strains) or resistant to a single antibiotic (20 strains) in a range of associated genotypes with no particular *cag*A or v*ac*A m-type predominant. Overall for Cla, the *cag*A frequencies were 42% (resistant) *vs* 68% (sensitive) whereas for Mtz, were 67% (resistant) *vs* 63% (sensitive). Likewise, for Cla, m2 frequencies were 53% (resistant) *vs* 56% (sensitive) and for Mtz, were 57% (resistant) *vs* 47% (sensitive). Interestingly, 71% (5/7) of the Cla resistant strains with the m2 genotype had an A2059G mutation. *hop*A, B, C and E were conserved in all strains except several gave an additional *hop*A amplicon.

The observed differences in genomic markers were consistent with the known genotypic diversity within *H. pylori*. For surveillance, the results indicated a need for additional genotypic markers linked specifically to resistance, particularly to Mtz resistance.

1620 Expression of the multidrug efflux gene *acr*AB during growth of *Escherichia coli* in nutrient-limited conditions

J.D. RAND, S.G. DANBY, D.L.A. GREENWAY and R.R. ENGLAND
University of Central Lancashire, Preston, Lancashire PR1 2HE

Multiple drug efflux pumps act as major factors to create the general intrinsic resistance of Gram-negative bacteria to natural and synthetic antibacterial agents. One such efflux system in *Escherichia coli*, encoded by *acr*AB-*tol*C operon, expels a range of antibiotics including, β-lactams, fluoroquinolones, novobiocin, tetracycline, erythromycin, and fusidic acid. It has been demonstrated that global environmental stress conditions, e.g. high osmolarity, fatty acids and 4% ethanol lead to an increased expression of *acr*AB-*tol*C. It is highly likely that global stress conditions cause a decrease in microbial growth rate, which in turn regulates transcription of efflux genes. In order to investigate this hypothesis, *E. coli* strains (differing in their ability to produce ppGpp and *rpo*S) were transformed with pNN602, (encoding *acr*AB::*lac*Z) and grown in batch and chemostat culture under varying nutrient conditions. Cells were analysed using Western blotting to determine sigma S production; HPLC to determine ppGpp levels; and beta-galactosidase measurements to monitor expression of *acr*A. Results from batch culture, in iron-limiting MOPS medium, show that expression of *acr*A in *E. coli* reached a peak at the end of exponential growth when the estimated growth rate was $0.4h^{-1}$. In chemostat culture, when the growth rate was set at $0.05h^{-1}$, expression of *acr*A was 3x greater than expression produced by *E. coli* grown at $0.3h^{-1}$. *E.coli* CGSC 805 (*rel*A1, *spo*T) transformed with pNN602, expressed *acr*A at the end of exponential growth in nutrient broth; induction of a high level of ppGpp [400pmol/A_{60}] in *E. coli* W4680 (pNN602) did not invoke increased expression of *acr*A. Western blotting showed that there was a correlation between increased amounts of sigma S and expression of *acr*A. However, *E. coli* JR23 (Δ*rpo*S) transformed with pNN602 was able to express *acr*A during batch culture in nutrient broth. We conclude that expression of *acr*A in *E. coli* is regulated by growth rate, and that regulation does not require the presence of ppGpp or sigma S.

1640 investigation of the association of anti-*Chlamydiophila (Chlamydia) pneumoniae* antibodies with anti-heart muscle antibodies

T.A. DILLANE[1], M. BAILLEY[1], N. LATIF[2], M.J. DUNN[2] and K.B. BAMFORD[1]
[1]Dept of Infectious Diseases and Microbiology, Imperial College School of Medicine, Hammersmith Campus, Ducane Road, London W12 0NN, [2]Heart Sciences Centre, National Heart and Lung Institute, Harefield Hospital, Harefield

It has recently been reported that anti-*Chlamydia pneumoniae* (Cp) antibodies cross-react with heart muscle and are associated with cardiomyopathy in mice.

To determine whether human sera that are anti-Cp positive have more antibodies that react with human heart muscle compared with those that are anti-Cp negative.

Human heart muscle, (Cp DNA negative) was extracted and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblots carried out using 128 sera selected from fit young(Y) (n=88 comprising 41 anti-Cp positive and 47 anti-Cp negative) and care of the elderly(E) (n=40, 20 anti-Cp pos, 20 anti Cp neg.) populations on the basis of their anti-CP status (IgG and IgA, detected by microimmunofluorescence, MRL Diagnostics). The number of sera with bands reacting with proteins of different molecular weights in the anti-Cp positive Y and E subgroups compared with the anti Cp-negative groups will be shown.

Of 20 different bands, 1-16 were detected in 127/128 samples. The band/sample ratio was 6.04 in the Cp negative group vs. 5.98 in the Cp positive group. Cp neg vs. Cp pos in the Y and E subgroups were 6.91 vs. 6.54 and 4.00 vs. 4.85 respectively. Initial analysis indicates that in this population possession of anti-Cp antibodies is not associated with an increase in the total number of different antibodies that recognise human heart muscle.

POSTERS

CM 01Species identification and Vancomycin resistance of clinical isolates provisionally identified as enterococci

A.M. DAY, J.H. COVE, J.A.T. SANDOE and M.K. PHILLIPS-JONES
Division of Microbiology, School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT

Enterococci are a significant and increasing cause of nosocomial infections. They have also become increasingly resistant to a range of antibacterial agents. Vancomycin resistance poses a particular problem due to the fear that such resistance may be passed from the enterococci to other Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus*, rendering infections caused by such organisms extremely difficult to treat. Here we report a survey on the identification and antibiotic resistance profiles of fifty nine isolates from patient samples taken in 1997 or 1998 at the Leeds General Infirmary, which had previously been provisionally identified as enterococci. We confirmed these isolates as enterococci and then identified them species level. The enterococcal isolates were tested for resistance to vancomycin and seven other antibiotics.

From our results we discuss the prevalence of *Enterococcus faecium* as an increasing cause of nosocomial infection and the incidence of vancomycin resistance amongst this small group of isolates.

CM 02Effect of clarithromycin treatment for the establishment of persistent macrolide resistant bacteria in the normal flora

GÖTE SWEDBERG, LARS ENGSTRAND, MARIA SJÖLUND, MARIA JÖNSSON and YVONNE QVARNSTRÖM
Dept of Pharmaceutical Biosciences and Dept of Medical Sciences, Uppsala University, Sweden

70 ulcer patients were treated for Helicobacter infection with a standard treatment including omeprazole, metronidazole and clarithromycin. Samples of bacteria from the normal flora were collected before treatment (A), directly after treatment (B) and one year after treatment (C). All samples were tested for the presence of bacteria resistant to clarithromycin.

In patients where resistant bacteria were found in samples collected one year after treatment, more detailed PCR analysis were performed to determine which genes where responsible for the resistance. Enterococci from faeces samples were predominantly carrying ermB, while streptococci from throat samples carried mefA genes and in addition one or several erm-genes. In Neisseria sp very few specific resistance genes were detected by the PCR primers used. A number of resistance genes were detected in samples collected before treatment, however samples collected after treatment showed a different pattern and generally a higher level of resistance.

Typing of individual bacterial clones isolated in A, B and C samples were performed by AP-PCR (DNA fingerprinting) to look for any changes in the population of bacteria following drug treatment. In general, the populations were homogenous before treatment but new clones were selected by the drug treatment and in some cases these persisted one year after treatment.

Conclusions: Genes conferring macrolide resistance are common in streptococci and enterococci belonging to the commensal bacterial flora. Drug treatment selects highly resistant clones with limited variation in the population. In some individuals, resistant bacteria with the same characteristics persist up to at least one year after treatment.

CM 03Activity of photobactericidal agents against drug-resistant bacteria

MARK WAINWRIGHT[1], DAVID A. PHOENIX[1] and DAVID R.A. WAREING[2]
[1]Centre for Forensic Science, University of Central Lancashire, Preston PR1 2HE, [2]Public Health Laboratory Service, Royal Preston Hospital, Preston PR2 4HT

A closely related series of phenothiazinium photosensitizers were tested for antibacterial activity against several pathogenic strains of *Staphylococcus aureus*, four of which were methicillin-resistant (MRSA). The photosensitizers were illuminated using a non-laser light source and this resulted in the enhancement of antibacterial activity in liquid culture. In several cases, illumination led to considerable decreases in the minimum lethal concentrations, giving increases in bactericidal activity of up to 16-fold. In addition, the greatest photosensitizer activity was exhibited against epidemic strains of MRSA, the photosensitizers being active at lower concentrations than vancomycin.

The toxicities and phototoxicities of methylene blue and its two methylated derivatives were measured against one standard and three vancomycin-resistant pathogenic strains of *Enterococcus* spp. Each of the compounds was bactericidal and the derivatives exhibited photobactericidal activity on illumination against one or more of the strains. It is proposed that increased bactericidal and photobactericidal activity in the methylated derivatives is due to their higher hydrophobicities allowing greater interaction with the bacterial cell wall. The possibility of employing these agents in local therapy or as disinfectants is discussed.

CM 04Susceptibility to para-chloro-meta-xylenol of a transient skin bacterium attached to skin squames

S.A. JOHNSON[1], P.S. HANDLEY[1], G. ROBSON[1], P. GODDARD[2] and B. TIMMINS[2]
[1]University of Manchester, 1.800 Stopford Building, Oxford Road, Manchester M13 9PT, [2]Reckitt-Benkiser Ltd, Dansom Lane, Hull

Carriage of pathogenic transient bacteria on the hands necessitates the use of skin disinfection products in medical and food production environments. Bacteria attached to surfaces as biofilms are considered to have an increased resistance to antimicrobial agents. It would be expected that resident bacteria, which can attach and multiply as microcolonies on the skin, would be more resistant to biocides *in situ* than transient bacteria, which are unable to colonise the skin. A model system using hand skin squames has been developed in order to determine the biocide susceptibility of bacteria attached to the skin surface. The model transient strain *Pseudomonas veronii* BL146 was studied. Skin squames obtained from the hands were UV irradiated to kill attached indigenous flora. Exponential and stationary phase bacteria were then attached to the squames under nutrient rich and nutrient limited conditions and challenged with PCMX. After biocide challenge D values were calculated and compared to D values for equivalent planktonic cells. Exponential planktonic cells were most susceptible to PCMX and there was an 11.8 fold increase in resistance for the most resistant starved planktonic cells. Transient bacteria would not be expected to form microcolonies on the squame and therefore would not exhibit biofilm properties such as increased resistance to biocides. This was shown with the model transient *Pseudomonas veronii* BL146. When attached to skin squames *Pseudomonas veronii* BL146 was significantly more susceptible to PCMX than the equivalent planktonic cells suggesting attachment conferred no increased resistance to PCMX.

CM 05Insertion elements and the expression of the carbapenemase gene (*cfiA*) in *Bacteroides fragilis*

R. EDWARDS
Division of Microbiology, University Hospital, Queen's Medical Centre, Nottingham NG2 7BL

Eleven IS element types have been detected in *B. fragilis* and some of these, typically IS*942*, IS*1186* and IS*4351*, have been reported associated with the carbapenemase gene, *cfiA*. In this study, we examined seven meropenem resistant clinical isolates of *B. fragilis* for IS elements upstream *cfiA* to elucidate the control of gene expression.

Specific meropenemase activities (SMA) of sonicates were measured spectrophotometrically. DNA adjacent to *cfiA* was amplified by PCR; primer 1 was designed from a conserved region upstream of *cfiA* and primer 2 from the sequence within *cfiA*. The size of the amplified PCR products was determined and the DNA sequenced. These were analysed using GenBank and the gapped-basic local alignment search tool (BLAST).

Two strains showed meropenem MICs of 16 mg/l, low SMA's and gave PCR products of 300 bp which did not contain IS elements. Five strains gave PCR products of 1.5-2.0 kbp which contained IS elements. Two of these strains showed MICs of meropenem of 64 mg/l, intermediate SMA values and possessed IS*1187* and IS*613*. The remaining three strains were highly resistant to meropenem, showed high SMA's and possessed IS*162* and elements 81% and 74% related to IS*942* and IS*1187* respectively.

Therefore, IS elements were associated with moderate to high *cfiA* expression and resistance, although low level resistance can occur in the absence of IS elements. Two of the IS elements encountered may represent novel types.

CM 06Sulphonamide resistance in *Haemophilus influenzae* mediated by *sul2* or an insertion in chromosomal *folp*

V.I. ENNE[1], D.M. LIVERMORE[2] and L.M.C. HALL[1]
[1]Dept of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine, Turner Street, London E1 2AD, [2]Antibiotic Resistance Monitoring and Reference Laboratory, CPHL

This study investigated the genetic mechanisms mediating sulphonamide resistance in *H. influenzae*. Eight highly sulphonamide resistant (MIC ≥ 1024 mg/l) *H. influenzae* isolates from the UK and Kenya were PCR positive for the *sul2* gene. *Sul2* encodes a drug-resistant dihydropteroate synthase enzyme and is frequent and often plasmid-mediated in enterobacteria. Plasmid extraction and Southern hybridisation confirmed that 7 of the PCR-positive haemophili carried *sul2* plasmids of various sizes. These plasmids also carried the streptomycin phosphotransferase genes *strA* and *strB*.

To determine the mechanism of sulphonamide resistance in *sul2*-negative isolates the chromosomal *folP* (dihydropteroate synthase) gene was sequenced from 3 resistant strains from the UK and Kenya and 8 susceptible strains from the UK. The *folP* sequences from the 3 resistant strains were identical and contained a 15 bp insertion encoding for Phe-Leu-Tyr-Asn-Asp after Pro-64, a residue that is highly conserved in all dihydropteroate synthases. By comparison, the 8 susceptible strains were diverse in their *folP* sequence and did not contain the insertion. Natural transformation of *H. influenzae* Rd with total DNA or the *folP* gene amplified by PCR from resistant strains raised the sulphonamide MIC from 8 mg/l to >1024 mg/l. A susceptible strain was then transformed to resistance with a shorter *folP* PCR product. The only coding difference between the recipient and the transforming DNA was the insertion, confirming that it is sufficient for mediating sulphonamide resistance in *H. influenzae.* The detection of both resistance mechanisms from different continents and over a period of 8 years implies that they are widely distributed in the species.

CM 07Antibiotic resistance in isolates of *Neisseria gonorrhoeae*: the London surveillance program

I.M.C. MARTIN, C.A. ISON, E. BURROWES and THE LONDON GONOCOCCAL WORKING GROUP
Dept Infectious Diseases and Microbiology, Imperial College School of Medicine, St Mary's Campus, London

Background: To determine the levels of antibiotic resistance to therapeutic antimicrobials in isolates of *N.gonorrhoeae* collected from 1997 to 2000, as part of the first surveillance program of gonorrhoea in London.
Methods: A single isolate of *N.gonorrhoeae* per patient was collected during a three-month study period from 1997 to 2000. Ten London genitourinary medicine clinics and their supporting laboratories were part of the collaboration, providing 75% coverage of gonorrhoea diagnosed in London and approximately 50% of all infections in England and Wales. Susceptibility of all isolates was determined to penicillin, ciprofloxacin, tetracycline, spectinomycin and ceftriaxone using standard methods. Limited demographic data was also collected.
Results: An increase of 77% in the incidence of gonorrhoea from 1997 to 2000 has been detected, with 1133 isolates tested in 1997 and 2015 in 2000. Plasmid mediated resistance to penicillin has increased over the four years from 0.5% to 2.9%. Plasmid mediated resistance to tetracycline has risen from 2.4% to 4.4%. Combined plasmid mediated tetracycline and penicillin resistance has also increased from 1.3% to 2.4%. Chromosomally mediated resistance to penicillin has decreased dramatically from 7.6% to 2.4%. Resistance to ciprofloxacin has increased from 0.4% to 1.0%. A rise in isolates with reduced susceptibility to ciprofloxacin has been detected from 0.5% to 2.5%.
Conclusion: During the large increase in incidence of gonorrhoea in London antibiotic resistance patterns have been changing, which may reflect changes in prescribing therapy. High level penicillin resistance in 2000 is now greater than 5%, a threshold suggested for considering alternative therapy. Reduced susceptibility to ciprofloxacin, the current alternative antimicrobial, over the past four years has also been increasing. It is important to monitor these changes through surveillance for informing therapeutic choice of antibiotics.

CM 08A membrane-bound ribonucleoprotein with cultural characteristics of a micro-organism and the resistance to inactivation characteristics of a TSE agent

D.W. BURDON
Dept of Clinical Microbiology, University Hospital Birmingham NHS Trust, Birmingham B15 2TH

A membrane-bound ribonucleoprotein (RNP) found in normal blood cells can replicate independently in cell-free media. The brown water-insoluble matter produced *in vitro* is composed of colourless particles containing RNA and protein which are capable of replication, co-aggregated with a pigmented iron-containing product derived from haemoglobin. The native membrane-bound particle has an additional protein which is absent from particles formed *in vitro*, and RNA is undetectable. The physiological function of the native RNP for which the generic name Ribonucleon is proposed might be involved with recycling haemoglobin iron within the extravascular compartment in association with macrophages.
Growth in agar produces an appearance resembling bacterial colonies. The viable particles within colonies are not inactivated by autoclaving at 134°C for 4 minutes, are resistant to formaldehyde and susceptible to hypochlorite and NaOH.

The properties of this RNP are such that if diverted from its normal physiological function by mutation, it would have the potential to behave either as the agent of an inherited disorder or as a transmissible agent.

CM 09Allelic variation in pneumococcal virulence determinants

I. BALAKRISHNAN, B. CHARALAMBOUS and S.H. GILLESPIE
Medical Microbiology Dept, Royal Free Campus, London NW3 2PF

Choline-binding protein A (CbpA) is a pneumococcal surface adhesin thought to play an important role in the adhesion of pneumococci to human cells. Under different names (PbcA, SpsA, PspC) different properties have been attributed to this molecule, including the ability to bind secretory IgA (SIgA). We have found allelic variation within the N-terminal portion of the CbpA gene that encodes the SIgA-binding region of the protein. In contrast, the autolysin gene, responsible for cell wall turnover, is far more conserved. The functional implications of these findings are being investigated.

CM 10Investigation of a cluster of isoniazid resistant mycobacterium tuberculosis in North London

MICHAEL RUDDY, SpR
Microbiology, Royal Free Hospital, London on behalf of the Outbreak Control Committee

The Microbiology Department of the North Middlesex Hospital in Edmonton North London noted 4 cases of smear positive pulmonary tuberculosis in young adults from the local population during January 2000. These were found to be isoniazid resistant and RFLP (restriction fragment length polymorphism) typing performed by the Mycobacterial Reference Unit revealed a similar 14 band pattern. Concerned about a possible outbreak in the local community a look-back exercise was performed on all isoniazid resistant cases over the preceeding 5 years and a further 9 cases were identified. A local Outbreak Control Group was formed and further case finding from the adjacent hospitals revealed 3 more. CDSC Thames were informed and a larger Outbreak Control Committee has been convened to investigate the cluster. To

date a total of 37 cases have been confirmed, mainly in the North London area but also involving other London districts.

We will describe the investigation process and the findings from the epidemiological analysis; discuss the common factors involved including imprisonment and drug use; consider the implications in terms of resources available to detect and manage such outbreaks. Finally to briefly consider the implications of the virulence or "fitness" of this isolate in terms of possible contribution to the number of cases.

CM 11Evaluation of PCR typing methods for methicillin-resistant *Staphylococcus aureus* (MRSA)

D. MORRISON, R. ADAM, G.F.S. EDWARDS, B. COSGROVE and C.G. GEMMELL
Scottish MRSA Reference Laboratory, Glasgow Royal Infirmary, Glasgow

Introduction: Pulse Field Gel Electrophoresis (PFGE) is the gold standard for typing MRSA, however, the equipment is expensive and the results are not obtained for several days. PCR based typing methods use less expensive equipment and the results are obtained in one day. The aim of this project was to evaluate several PCR methods for typing MRSA.
Methods: A set of 16 MRSA clones characterised by phage typing, biotyping, antibiograms and PFGE were used to evaluate eight PCR methods: IS256, RW3A icaC/geh (IG), uvrA/hprK (UH), STAR (ST), Tn916/Shine Dalgarno (TS), Coagulase and Protein A. Several DNA extraction procedures and PCR reagent mixtures were compared.
Results: Four of these typing methods (ST, UH, TS, and IS256) gave poor results by performance criteria. IG produced only four types. RW3A was the most discriminatory single typing method dividing the 16 clones into 12 types. Protein A (8 types) was more discriminatory than Coagulase (3 types), however, RFLP of the Coagulase gene product by two restriction enzymes was as discriminatory as the RW3A typing method.
Conclusion. The RW3A PCR typing method using purified DNA and PCR Ready-To-Go Beads is a useful alternative for typing MRSA

CM 12PCR based identification of Coagulase Negative Staphylococci (CNS) to the species level

K. GIRVAN[1,2], K. LIDDELL[2], D. MORRISON[1], G.F.S. EDWARDS[1], B. COSGROVE[1] and C.G. GEMMELL[1]
[1]Scottish MRSA Reference Laboratory, Glasgow Royal Infirmary, Glasgow, [2]Microbiology Dept, Law Hospital, Lanarkshire

*Introduction***.** Identification of CNS to the species level by standard phenotypic methods is often problematic. The aim of this project was to compare 16S-23S PCR (de Baere *et al*, Abstract 1573, ICAAC 1999, San Francisco, USA), involving amplification of the 16S-23S rRNA spacer region, and several phenotypic methods.
Methods. A set of 45 clinical and reference isolates of CNS, comprising seven species, were identified by three phenotypic methods (API STAPH, ID 32 STAPH and an In-House), 16S-23S PCR and CNS species specific primer PCR (SS PCR) published by Gribaldo *et. al*. 1997, J. Med. Micro. 45: 45-53. The SS PCR was regarded as the "gold standard" identification in this study.
Results. The patterns generated by the 16S-23S PCR agreed completely with the species SS PCR identification. The phenotypic methods identified to species level with varying degrees of accuracy: API STAPH (84%), ID 32 STAPH (69%) and In-House (56%). By the 16S-23S PCR small variations were observed in intra-species patterns, however, these were easily distinguished from inter-species patterns. 16S-23S PCR is more cost effective then SS PCR as it requires only one PCR amplification per isolate.
Conclusion. PCR amplification of the 16S-23S rRNA spacer region is a useful alternative method for species identification of CNS.

WEDNESDAY 28 MARCH 2001

0900 Clinical Virology

J.E. BANATVALA
Emeritus Professor of Virology, St Thomas's Hospital, London

One of the key figures in the development of Clinical Virology was Professor A.P. Waterson. He developed an interest in the clinical applications of Virology in the early 1960's whilst working in the Department of Pathology in Cambridge, where most of the work was then related to basic science rather than its' clinical applications.

Tony Waterson became Professor of Virology at St Thomas' Hospital Medical School (1964 – 67), transferring to a Professorship at the Royal Postgraduate Medical School (RPMS) in 1967 until he died in 1983. In both these Medical Schools he fostered the development of Clinical Virology and whilst at RPMS arranged meetings in Clinical Virology attended first by merely a handful of those with an interest in Clinical Virology. However, within a short time, meetings became much larger and filled one of the major lecture theatres. Subsequently, as Clinical Virology developed, the size of meetings became even larger and the venue was transferred to the Charing Cross Hospital Medical School, the meetings then being organised by Professor Jonathon Coleman.

It soon became apparent that these increasingly popular meetings required some form of organisational infrastructure and an approach was made by Professor Peter Wildy and Dr. John Beale, President and Treasurer respectively of the Society of General Microbiology, for the Clinical Virology meetings to be incorporated into the SGM. There was some opposition to this since it was felt that the Group might lose its autonomy but the attraction of having the SGM's professional secretariat, then based at Harvest House in Reading, together with the SGM's secure and considerable funding, resulted, after a vote, in the Clinical Virology Group being incorporated into the SGM.

The first Convenor of this Group was Professor J.E. Banatvala, followed by Professor Coleman and the first meeting was held at Churchill College, Cambridge. A large number of Clinical Virologists attended and as the lecture theatre could not accommodate them all, the first meeting had to be moved to the Cricket Pavilion.

In the early 60's, Clinical Virology was still in its infancy, but the during this decade, the development of techniques for the virological diagnosis of intrauterine and perinatal infections such as rubella, began to make an impact on the diagnosis and management of patients. The specific IgM tests which were developed, were then applied to a number of virus infections, thereby speeding up laboratory diagnosis, and not having the necessity to test paired sera. Perhaps the next major development related to the identification of the so-called Australia antigen and its relationship to infection by hepatitis B. Early techniques were somewhat insensitive, but by the 1970's, most laboratories were employing sensitive and specific radioimmunoassays. During this and subsequent decades, Clinical Virologists have increasingly been involved in the diagnosis and management of virus infections in the immunocompromised, particularly patients who have had organ transplants, and, of course, HIV. They have been increasingly involved in research and diagnosis of sexually transmitted diseases. Clinical Virologists have also been to the forefront in providing advice, not only for their own Laboratories, but also other specialities in Clinical Pathology, on various aspects of laboratory safety. In addition, today's Clinical Virologist has taken up the initiative, perhaps more so than any other branch of Clinical Pathology, developing skills in molecular biology; molecular diagnostics is now very much part of the diagnostic repertoire.

0930 Management of hepatitis

H. THOMAS
Imperial College School of Medicine at St Mary's, London

Abstract not received

1015 Hepatitis B and health care workers

WILLIAM F. CARMAN
West of Scotland Regional Virus Lab

There are three major issues. First, protecting health care workers against hepatitis B. This is part of a failed strategy to selectively immunise the British population, although it has been very successful in health care workers. Arguments for universal vaccination will be briefly presented. Second, we need to protect patients from becoming infected by health care workers. Vaccination of health care workers is again the core of this, but there are also policies of not allowing infected health care workers to perform exposure prone procedures (EPP). Until recently, it was only HBeAg positive persons who were forbidden from undertaking EPP, but this has changed with the dawning of a realisation that anti-HBe positive persons can also be viremic, and infectious. This links in with the recent guidelines issued by the Department of Health regarding anti-HBe positive carriers and EPP. A titre of 10^3 genomes per ml of blood is now the cut off for proceeding with EPP; a level above this and the health care worker has to find another job. The rationale for this and the current results will be presented. However, it is also critical that if a health care worker who is known to be HBsAg positive, if allowed to operate, lets a significant amount of blood into a patient, that there should be reporting of the incident to the appropriate authorities, as it is the volume of blood as well as the virus titre which can influence the chance of infection of a patient. The third issue is protocol for follow-up of vaccination of health care workers; I will present the newly introduced simplified Scottish protocol. Overall, I will try to answer the question: "is the British approach to HBV and health care workers over the top?"

1130 Detection of intra-hepatic HCV-specific CD8 positive T cells by MHC-peptide tetramers

A. GRABOWSKA[1*], F. LECHNER[2], P. KLENERMAN[2], P. TIGHE[1], S. RYDER[3], J. BALL[1], B. THOMSON[1], A. ROBINS[1] and W.L. IRVING[1]
School of [1]Clinical Laboratory Sciences and [3]Medicine, Nottingham University, [2]Nuffield Dept of Medicine, University of Oxford

Background: Hepatitis C virus (HCV) is an important cause of post-transfusion and community-acquired hepatitis. In spite of the importance of HCV as a human pathogen, the mechanisms leading to liver damage in infected patients are not well understood. The liver of hepatitis C-infected patients is characterised by a mononuclear cell infiltrate. Determination of the proportion of intrahepatic lymphocytes in HCV-infected livers which have specific reactivity against the virus is a crucial issue in understanding the potentially beneficial role of the immune response to the virus and the pathology associated with infection. With the advent of the tetrameric MHC-peptide complex technology it has become possible to directly determine the specificity of the antigen-specific receptor of $CD8^+$ T cells in combination with other phenotypic markers. Methods and *Results:* We have separated CD8 cells from HCV-infected livers from patients at an early stage in disease. Using a panel of HCV-specific tetramers, we have shown that there is a raised proportion of HCV-specific intrahepatic CD8 lymphocytes compared with CD8 cells from the periphery of the same patients, and that these cells have recently been activated. Such cells are not observed in the liver of uninfected patients or in infected,

HLA mismatched controls. These results are in contrast with other studies which have not detected such cells in late-disease-stage livers. Future studies will involve a detailed characterisation of functional markers on these cells.

1215 The role of antiviral resistance testing for HIV

C. LOVEDAY
Royal Free and University College Medical School, London

Abstract not received

1415 Management of needlestick injuries

M.E. RAMSAY
PHLS Communicable Disease Surveillance Centre, London

Abstract not received

1500 Paediatric HIV, presentation and prevention

HERMIONE LYALL
Imperial College School of Medicine at St Mary's, London

Epidemiology

The most recent epidemiological data (1999) shows that prevalence of HIV infection in pregnant women is at the highest level recorded, with 1 in 400 women in London infected. Prevalence in London varies substantially according to area of residence, from 1 in 120 to none. Elsewhere in the UK antenatal sero-prevalence remains low (1 in 4,500). Although antenatal testing is increasing and 76% of maternal infections were detected in pregnancy in London in 1999, a similar increase in antenatal detection has not yet been seen in the rest of the country. Antenatal detection rates should continue to improve as hospitals implement new testing policies.

Up to Oct 2000, 740 HIV infected children had been reported in the UK, of whom 539 live in the Thames regions. 12% of these children were born abroad, more than 80% were born to mothers of African origin, in the vast majority of cases diagnosis of infant / maternal infection was made after birth.

Timing of infection and Prevention of Transmission

As with the other blood borne infections, the majority of HIV transmission from mother to infant occurs around the time of delivery, with very little in the first two trimesters of pregnancy. Where a mother breast feeds this will further double her risk of transmission. The single most important factor for an individual women in terms of her risk of transmission is her plasma viral load: for women with a viral load of >100,000 copies / ml the transmission risk is >60%; for women with <1000 copies / ml the risk is very low, <1%. Other factors which increase the risk of transmission include: primary infection during pregnancy / breast feeding; advanced maternal disease; other sexually transmitted diseases; illicit drug use in pregnancy; premature delivery (more common in women with advanced disease); labour; duration of ruptured membranes; and interventions during labour.

Interventions to reduce transmission have been highly successful and include a combination of approaches tailored to an individual women's needs. Formula feeding is advised in the UK. Women with advanced disease / high viral loads need combination antiretroviral therapy (ART) for their own disease, the corollary of effective treatment being control of virus with reduced risk of transmission. Caesarean section reduces the risk of transmission at all levels of maternal viral load, whether it has anything to add when the viral load is undetectable is not known. Thus women as making effective choices about ART, mode of delivery, and infant feeding to greatly reduce the risk of transmission. Use of ART in pregnancy is being monitored for possible long term effects (eg teratogenicity, carcinogenicity, mitochondrial toxicity), to date no overall increases have been demonstrated.

HIV infected children, how do they present?

Paediatric HIV disease is a rare condition in the UK, but may present in a number of common ways to any children's service. The spectrum of disease includes approximately 20% of children who present within the first year of life with severe disease (eg primary pneumocystis pneumonia, failure to thrive, orencephalopathy) and the remainder who may present at any age up to the teens. These less severely affected children may have chronic parotitis, lyphadenopathy and lyphoid interstitial pneumonitis, recurrent infections, chronic diarrhoea etc. There is an HIV classification system for children based on symptoms of disease and age adjusted CD4 counts. Viral loads may be particularly high in infancy as the infant immune system does not bring the virus under control as quickly as is seen in adults.

Principles of treatment are the same for children as infected adults, but there are major problems of formulation and tolerability for children and not all the medications available for adults are suitable for children. Adherence to treatment in children needs a family centred, multidisciplinary approach. There are concerns about toxicities in children exposed to ART for many years, so close follow up is important. Immune reconstitution after starting treatment may be more effective in children that adults as a result of the immaturity of the system. There is an increasing cohort of vertically HIV-infected adolescents who have particular needs in terms of treatment and support.

1600 Blood borne viruses and the safety of blood products

JOHN BARBARA
National Blood Service - North London, Colindale Avenue, London NW9 5BG

Concerns about the microbiological safety of blood products are twofold. First, 'fear of the unknown' i.e. newly recognised agents such as GBV-C, TTV, SEN-V, HHV-8 and the more sinister vCJD. Second, somewhat unrealistic fears of remaining risks from agents such as HIV, HBV and HCV for which a range of 'high tech' interventions are already in place and have been demonstrated to be highly effective.

The 'new' agents such as GBV-C appear to be non-pathogenic and although SEN-V may be implicated in some cases in Dr. Alter's panel of PT-Non A to E samples, residual PTH of any kind in the UK is extremely rare. HHV-8, the causative agent of Kaposi's sarcoma has so far not been shown to be transmitted when recipients of cellular components prepared from anti-HHV-8 positive donors are followed up. Variant CJD has likewise not been shown to be transmitted by blood transfusion. However, because of the theoretical risk associated with this disease, major initiatives such as leucodepletion of all blood components and exclusion of UK donors from donating plasma for fractionation, have been implemented.

Absence of transmission by blood of the 'established' microbial agents can be calculated or demonstrated as follows:

1. Direct testing of blood recipients as in the North London Transfusion Transmitted Infection (TTI) study which showed no transmission of HIV, HBV, HCV or HTLV from 22,000 blood components. Indeed, residual risk is now so small for HIV and HCV that prospective studies of recipients to detect infection would be impracticably large. The risk of transmission of HBV by low-level carriers is however likely to exist: calculations of a risk level of up to 1 in 50,000 are supported by a recent study by Professor Allain *et al*.
2. Risk can be calculated from the product of the 'window period' prior to seroconversion and the incidence of an infection in blood donors, together with test and test processing errors. This had been estimated at 1 in 250,000 for HCV an 1 in 2.8 million for HIV (Soldan and Barbara). However, automation, computerisation, effective process control and enhancements of assays are likely to make these figures overestimates.
3. Assays such as PCR to directly detect viral genomes support the concept of lower actual risk than calculated. Thus only 3 donors in the UK Blood Services have been found to be HCV RNA positive prior to seroconversion, in 3 million donations tested in pools of 96 samples.

4. The ultimate test (albeit usually only for symptomatic infections) of safety is the collation of reliably investigated reports of post-transfusion infections. This is done nationally through SHOT (Serious Hazards of Transfusion). In the last 3 years there has been 1 case of HAV, 1 donor infectious for HIV (the only one in England since anti-HIV screening started in 1985) and one or two cases per annum of HCV and/or HBV, with approximately 3 million blood donations annually. However, there was 1 fatal transmission of *Plasmodium falciparum* and approximately 3 transmissions of bacterial infection per annum. These reflect a range of species and often prove fatal. They highlight the need for more effective action against bacterial risk and this is now being developed.

Currently a wide range of new initiatives including enhanced serological screening, introduction of PCR, universal leucodepletion, viral inactivation, out-sourcing plasma for fractionation and several bacterial risk reduction strategies are either being implemented or assessed. It remains to be seen which prove most cost-effective. Undoubtedly however, an already safe blood supply is being made even safer although it may prove difficult to measure the increment.

1645 Blood borne viruses and the safety of organ transplantation

TIM WREGHITT
Clinical Microbiology and Public Health Laboratories, Addenbrooke's Hospital, Cambridge CB2 2QW

There are several blood-borne viruses which may be transmitted to transplant recipients via the transplanted organ(s). These include hepatitis B virus (HBV), hepatitis C virus (HCV), HIV, human parvovirus B19 and HTLV-1.

Based on risk assessment in the UK, human parvovirus B19 and HTLV-1 transmission is regarded as sufficiently more to justify testing all organ donors. However, there are many reports documented HBV, HCV and HIV transmission organs from HBsAg, HCV antibody and HIV antibody positive donors transmit infection to the recipient in almost 100% of cases and in the UK testing for these three viral markers is mandatory. Many donor virology tests are conducted outside normal laboratory working hours and confirmatory tests are necessary to confirm the status of donors who are reactive in HBsAg negative but anti-HBc positive may transmit HBV infection to liver recipients. In the USA, transmission has been reported in 80% liver recipients.

In the UK, insufficient denominator studies have been conducted to determine the accurate risk of infection in recipients of livers from HBsAg negative, anti-HBc positive donors. However, livers from HBsAg negative, anti-HBc positive donors are recommended to be transplanted only to HBsAg positive or HBV immune recipients or those who are in urgent need of transplantation and for when no other organs are available.

CMV and EBV can also be transmitted via blood and organs to transplant recipients.

THURSDAY 29 MARCH 2001

0900 No fall in HIV incidence in homo-/bisexual men since advent of highly-active antiretroviral therapy

G. MURPHY, J.V. PARRY, C. GRAHAM, C. McGARRIGLE and N. GILL
Central Public Health Laboratory, London

Background: The determination of HIV incidence is important for tracking current transmission patterns. This provides important evidence about changing patterns of risk-taking and the effectiveness of preventive measures. However, epidemiologically valid direct measures of HIV incidence are difficult to obtain. A novel laboratory technique is now available which can, when applied to individual anti-HIV positive specimens, distinguish incident from prevalent infections. This technique lends itself to application to specimens collected in unlinked anonymous HIV surveys.

Study Population: Homo-/bisexual men (n=19,832) who attended fifteen clinics participating in the Unlinked Anonymous HIV Prevalence Monitoring Programme between 1995 and 1998, and who had a syphilis test.
Method: An aliquot of specimens provided for syphilis testing was archived for surveillance of HIV infection. The specimens were unlinked from personal identifiers, but retained linkage to selected demographic and clinical information. All eligible specimens were screened for anti-HIV, and reactive specimens underwent confirmatory testing at the Virus Reference Division. The serological testing algorithm for recent HIV seroconversion (STARHS) was applied to anti-HIV positive specimens. Specimens from men whose infection had previously been diagnosed were excluded from the final analysis because of the potential confounding effect of highly active anti-retroviral therapy (HAART). The annual incidence was calculated after adjustment for missing specimens and repeat attendance rates at the clinics. Standard statistical analyses were applied to the findings.
Results: During the period 1995-98, 749 specimens from homo-/bisexual men whose HIV infection had not previously been diagnosed were shown to be anti-HIV positive. The STARHS assay indicated that 117 (16%) of the 749 infections were recent. The annual incidence for each year was around 2%, and did not vary significantly over the four year period.
Conclusions: Our results show a substantial HIV incidence occurring in homo/bisexual men attending GUM clinics and the need for increased behavioural interventions in this group. This level of HIV incidence is continuing despite widespread availability of HAART.

0915 Characterisation of diverse human immunodeficiency virus type 1 sequences using phylogenetic trees, similarity plots and bootscanning

I.D. TATT[1,2], K.L. BARLOW[1], P.A. CANE[3], D. PILLAY[3] and J.P. CLEWLEY[1]
[1]Sexually Transmitted and Blood Borne Virus Laboratory, Central Public Health Laboratory, London, [2]Institute of Public Health, Department of Public Health and Primary Care, University of Cambridge, Cambridge, [3]PHLS Antiviral Susceptibility Reference Unit, Division of Immunity and Infection, University of Birmingham Medical School, Birmingham

Human immunodeficiency virus type-1 (HIV-1) generates extensive diversity by the accumulation of point mutations and by recombination. Such diversity has the potential to affect phenotypic properties of the virus which may influence the design of vaccines and diagnostic tests. Given the extensive and continued global spread of HIV-1 it is necessary to characterise circulating virus strains as fully as possible. Here we describe the use of similarity plots and phylogenetic analyses to determine the extent of genetic diversity within non-subtype B strains in the United Kingdom. Twenty-two samples were identified as intersubtype recombinants in either *gag*, *pol*, and/or *env* by phylogenetic analysis. Further investigation of these samples using similarity plots and bootscanning identified a high level of intragenic genetic diversity in 18 of these mosaic genomes. Phylogenetic reconstruction of the potential intragenic recombinant breakpoints provided support for this high level of diversity. All but one of the 18 samples that showed potential intragenic recombination breakpoints had at least one region similar to a subtype A virus. These findings support the previously observed high level of genetic variation within lentivirus genomes, indicating a relatively high frequency of recombination.

0930 A pilot study of the prevalence of genotypic markers of resistance to protease inhibitors in treatment naïve and experienced HIV-1 infected individuals in South Wales

C. MOORE, S. CORDEN and D. WESTMORELAND

Dept of Medical Microbiology and Public Health Laboratory, University Hospital of Wales, Heath park, Cardiff
CF14 4XW
Protease inhibitors (PI) are an integral part of potent HAART. HIV drug resistance has been described for all classes of ART and its role in treatment failure is of increasing concern. The protease region of HIV is highly polymorphic and minor mutations within the protease enzyme are reported in treatment naïve individuals. It is not clear what the prevalence of such markers is in the UK HIV infected population and their correlation with anti-retroviral therapy and treatment failure. This study provides insight into the prevalence of resistance markers to PI in HIV infected individuals in South Wales. Patient groups studied include:

- Patients who are treatment naïve (n=20).
- Patients who are taking a PI (n=20).
- Patients who have taken a PI in the past (n=20).

HIV RNA was extracted from plasma using the QIAamp RNA mini kit (Qiagen). The protease region was amplified by reverse transcription (Access RT kit, Promega) and semi-nested PCR using published primers. The PCR product was run on a 1.5% gel and visualised using sybr gold post staining. The product was purified using the QIAquick PCR purification kit (Qiagen) and DNA yield estimated by comparison with known amounts of lambda DNA. Sequencing was performed on the CEQ-2000 Automatic Sequencer (Beckman Coulter). Following initial analysis in Cardiff, the sequence data was further analysed using the Stanford resistance sequence database.

0945 Antenatal HIV screening target met in Lothian, Scotland

M.M. OGILVIE, G.V. MAY, M.J. SHEARMAN, J.F.D. PRYDE and S.M. BURNS
Regional Clinical Virology Laboratory, City Hospital, Edinburgh EH10 5SB
UK Departments of Health set targets for the offer and recommendation of an HIV test to all pregnant women by December 2000, with uptake of at least 50% increasing to 90% by 2002 so that minimum 80% HIV infected are offered treatment to reduce vertical transmission. Lothian Health introduced an opt-out scheme, based on previous experience in this region where prevalence of HIV in pregnancy was 0.1% in 1998, starting 1st December 1999. Information leaflets were issued with booking appointments, consent recorded, and single serum tested in simultaneous (semi-automated) assays for HIV, HBV, rubella and syphilis.

Interim analysis showed overall 95% acceptance of all four tests, with some variation between community (92%) and hospital (96%) locations. New HIV diagnoses were made in 3 women within the year to end November 2000. With known positives having antenatal care, this gives the same prevalence as in 1998.

Women accept an HIV test offered as routine antenatal care, with less than 5% opting out when booked by most midwives. The variation between community and hospital may be addressed by further educational sessions for midwives.

1000 Herpes simplex encephalitis: involvement of apolipoprotein E genotype

M.A. WOZNIAK[1], W.-R. LIN[1], M.M. ESIRI[2], P. KLENERMAN[3] and R.F. ITZHAKI[1]
[1]Dept of Optometry and Neuroscience, UMIST, Manchester, M60 1QD, [2]Dept of Clinical Neurology, University of Oxford, and Oxford Radcliffe NHS Trust, Oxford OX3 9DU, [3]Nuffield Dept of Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU
We established, using polymerase chain reaction (PCR), that a high proportion of brains of Alzheimer's disease (AD) patients and of age-matched normals harbour latent herpes simplex virus type 1 (HSV1) in brain. We subsequently found that HSV1 in brain of carriers of the type 4 allele of the apolipoprotein E gene (apoE-ε4) is a strong risk factor for AD, and that neither HSV1 nor apoE-ε4 alone is a risk (Itzhaki et al., 1997; Lin et al, 1998) (and that apoE-ε4 is a risk factor for herpes labialis). We suggested that the virus in apoE-ε4 carriers is particularly harmful in the nervous system and that in their brains it reactivates periodically, causing localised damage - and eventually AD.

The aim of this study was to find if an apoE allele is involved in another HSV1 disorder, herpes simplex encephalitis (HSE).

We investigated apoE genotypes of DNA from fixed tissue of 14 HSE patients, confirmed by necropsy, and from the cerebrospinal fluid of 7 patients with HSV1 in their CSF detected by PCR. Genotypes were determined by semi-nested PCR or by one-step PCR, followed by restriction nuclease digestion. We found no difference in apoE-ε3 and apoE-ε4 frequencies between HSE patients and normal subjects. However, the apoE-ε2 allele frequency of the HSE patients was significantly higher than normal (OR=4.6, 95% CI, 2.0-10.8). We conclude that apoE-ε2 is a risk factor for HSE.

1015 Audit of the use and effectiveness of zoster immune globulin in a Welsh population

RACHEL N. JONES, DIANE McCRACKEN and DIANA WESTMORELAND
Cardiff Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff
Background and objectives: The use of human varicella-zoster immunoglobulin (VZIG) is advocated for the prevention or attenuation of primary varicella infection in certain risk groups following contact with the virus. There is very little published information on the efficacy of this passive antibody and none at all that relates to the Welsh population. At Cardiff PHLS we decided to audit the use of 'VZIG' to determine patient groups receiving the antibody and whether it was effective.
Methods: The audit began in July 1996 and has continued to date. Data presented represents the period July 1996 – Dec 1998. Clinicians requesting 'VZIG' were asked to complete a questionnaire 4 weeks after the administration of 'VZIG'. Questions addressed in the audit were:

Identification of patients groups to whom 'VZIG' was issued.

Time period between contact and receiving 'VZIG'.

Number of patients that developed clinical infection after receiving 'VZIG'.

Results: 69 doses of 'VZIG' were issued during this period, 47% of recipients were pregnant contacts of varicella, 40% were immunocompromised and 9% were neonates. Twenty six questionnaires were returned reporting 7 patients who developed chicken pox after receiving 'VZIG'.
Discussion: The safety of blood products has caused concern recently and stocks of 'VZIG' are in short supply. Improved therapy options for the prevention of primary varicella infection in risk groups will be explored.

1100 The use of real time PCR for the detection of CMV DNA in three patient groups

SALLY CORDEN and DIANA WESTMORELAND
Dept of Medical Microbiology and Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff
CF14 4XW
Objectives: Real time PCR provides a rapid diagnostic tool for the detection of several targets including CMV. In Cardiff the use of real time PCR for the detection of CMV DNA in three major patient groups, paediatric (urine samples), antenatal (amniotic fluid) and immunocompromised (respiratory samples) is currently under investigation.
Methods: Real time PCR was used in the detection of CMV DNA in 30 amniotic fluid samples, 49 urine samples and 15 respiratory samples. Inhibition was controlled for by spiking

all negative samples with a known amount of control CMV DNA.
Results and Conclusion: The results show that in 5 respiratory samples and 2 urine samples CMV DNA was detected. The 2 urine samples were also shown to be positive by cell culture methods. One of the 5 positive respiratory samples was also found to be positive by nested PCR only. Inhibition was only found in 2 samples and was easily removed by diluting the samples 1 in 10. We have found that detection of CMV DNA by real time PCR is both rapid and sensitive for the patient groups studied. Rapid and sensitive detection of CMV DNA enables early therapeutic intervention where CMV disease is suspected.

1115 Molecular epidemiology of rotaviruses in the UK 1995-98: frequency and analysis of mixed infections and reassortants

MIREN ITURRIZA-GÓMARA, ULRICH DESSELBERGER and JIM GRAY
Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QW

The G and P types of 3601 rotavirus strains collected in the UK between 1995 and 1999 were determined. In 95.4% of isolates the most common G/P combinations were encountered: G1P[8], G2P[4], G3P[8] and G4P[8]. Of the remaining a small but significant number were alternative combinations of the most commonly cocirculating strains (1.4% of the total): G1P[4], G2P[8] and G4P[4]. There were also strains of common G types with uncommon P types (0.6% of the total): G1P[6], G1P[9], G3P[6], G3P[9], G4P[6], and of uncommon G types with common P types (2% of the total): G8P[8] and G9P[8]. Partial sequencing of the VP7 and VP4 genes and phylogenetic analysis strongly suggested that these strains were reassortants between commonly cocirculating human rotaviruses, or with rotaviruses from other sources. Dual infections, characterised by the presence of 2 different G and/or P types in the same sample were found predominantly in those seasons were the diversity of cocirculating rotavirus strains was greater. Associations between the VP4 and VP7 genes of different P[8] lineages with particular G types were found, which require further exploration. These data suggest that genetic interaction by reassortment among cocirculating rotaviruses is a relatively frequent event, and contributes significantly to their overall diversity.

1130 Use of PCR for virological monitoring of respiratory illness in the community as part of an enhanced influenza surveillance scheme in Scotland

L.A. WALLACE[1], W.F. CARMAN[1], A. SMITH[2] and P. CHRISTIE[2]
[1]Regional Virus Laboratory, Gartnavel General Hospital, 1053 Great Western Road, Glasgow G12 0YN and [2]Scottish Centre for Infection and Environmental Health, Clifton House, Clifton Place, Glasgow G3 7LN

The annual outbreak of influenza in Scotland is monitored by sentinel general practices using a clinical diagnosis of influenza-like illness. Over the past two winters we have shown that a multiplex reverse-transcription polymerase chain reaction (RT-PCR) assay can be used successfully to provide real-time surveillance of community respiratory illness. Our PCR assay can be used as an early warning system to confirm the presence of influenza in the community.

This year, in a Scottish Office funded project to develop an improved influenza surveillance scheme, we are using PCR to screen combined nose and throat swabs from persons presenting to their general practitioner with a respiratory illness. The samples are tested for influenza A, influenza B, respiratory syncytial virus (RSV) and picornavirus in a multiplex assay; in addition an adenovirus PCR and a parainfluenza RT-PCR are also being used on all samples negative in the multiplex reaction.

The scheme will provide data sets that are more representative of the Scottish population, and will now include clinical data linked to the laboratory result. The production of data that is more accurate and timely will allow for strategic planning to ease the winter influenza crisis in the NHS.
To date, 27% of the samples sent are picornavirus positive and 7% RSV positive. Our previous experience shows that picornavirus, ie rhinovirus, will continue to be the most significant cause of respiratory illness until influenza starts to spread in the population.

The final results will be available at the end of the winter respiratory season.
(We thank the Scottish Office for their funding and all the general practioners for their cooperation.)

1145 Development of an immuno-PCR assay for the detection of mumps-specific IgG

ANNE M^{c}KIE, DHANRAJ SAMUEL, BERNARD COHEN and NICHOLAS SAUNDERS
Virus Reference Division, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

The serological detection of mumps-specific IgG plays an important role in immunity surveillance, including the monitoring of the efficacy of vaccination programmes, the identification of susceptible cohorts in the population and the shaping of future immunisation policies. Immunoassays are the method of choice for viral antibody detection but there is concern that they may lack the sensitivity required for certain applications, such as determining the immune status of individuals where natural or vaccine exposure may have been many years past.

We have developed an indirect, quantitative immuno-PCR assay for the detection of antibody to mumps virus, which addresses the problem of sensitivity. Serum samples were reacted with immobilised recombinant mumps antigen and bound antibodies were detected by PCR, using a conjugate of anti-human IgG covalently coupled to a short oligonucleotide (=capture probe). Target DNA, designed to hybridise to capture probe, was released into solution by restriction digest and then quantified using real-time PCR. The amount of target DNA was proportional to the level of specific antibody in the specimen.

We found the sensitivity (and specificity) of the immuno-PCR assay did not exceed that of conventional ELISA. The sensitivity was limited by non-specific binding of human IgG to the solid phase. Further development of reagents and assay formats is necessary to fully exploit the potential of quantitative I-PCR, so that potential improvements in sensitivity of anti-mumps IgG detection can be realised.

1200 Human papillomavirus detection and typing; An adjunct to cervical screening

S. STRAUSS, U. DESSELBERGER and J. GRAY
Clinical Microbiology and PHL, Addenbrooke's Hosp, Hills Road, Cambridge CB2 2QW

Human papillomaviruses (HPV) are the commonest sexually transmitted disease world-wide. Several outcomes of infection with HPV are possible, including: complete resolution and elimination of the viral infection, persistence with no cytological abnormalities, transient cytological abnormalities, persistent cytological abnormalities, or abnormalities which progress to carcinoma *in situ* or invasive cancer. Currently, HPV testing is not included in the National Cervical Screening Program. Major studies are underway to determine the role of HPV testing as an adjunct to cytological examination.

HPV DNA in genital samples was detected by a nested PCR with the established MY09/MY11 and GP5+/GP6+ primers. PCR amplicons were typed by reverse hybridisation with labelled type-specific probes.

Preliminary data obtained by the PHLS Human Papillomavirus Reference Laboratory would suggest that;
1. HPV DNA detection in women over 35 years of age is more likely to be associated with high risk HPV types (16,18 etc.). The absence of HPV types (6, 11 etc.) associated with benign lesions in this age group suggests persistent infection with high-risk types rather than continued acquisition of types through sexual activity.
2. Samples deemed inadequate for cytological examination (approx. 5 - 15% of total cervical smears examined) may be appropriate for HPV PCR; 72% of inadequate samples were adequate for HPV DNA detection.
3. HPV DNA testing was often able to elucidate the reasons for lack of correlation between abnormal cytology and colposcopy; HPV DNA was detected in 39.1% of women in whom no obvious colposcopic lesions were detected, compared with 81.4% with lesions.

These results suggest that HPV detection and typing may have a limited but important role in cervical screening. Algorithms, utilising and combining the best attributes of cervical cytology and HPV detection, recommending the periodicity of screening in defined cases and including guidelines for patient management are needed.

1215 Comparison of strand displacement amplification (SDA) with ligase chain reaction (LCR) for Chlamydia trachomatis in urine samples and endo-cervical swabs

R.A. McCARTNEY, J. WALKER and A. SCOULAR
The Regional Virus Laboratory, Gartnavel General Hospital, 1053 Great Western Road, Glasgow G12 OYN

Molecular amplification techniques for the diagnosis of genital *Chlamydia trachomatis* infections have improved the detection rate and allow non invasive urine samples in female as well as male patients to be used.

In this study we compared the novel SDA test (ProbTek from Becton Dickinson) with the LCx assay (Abbott Laboratories) on male urine and female urine and endocervical swabs from a genitourinary medicine clinic. Where discordant results were found an in-house nested PCR test was used to determine the "true" result of the sample. Each test was then measured against this "true" result, i.e the result of any two molecular tests.

Results. In male urine samples the sensitivity was 95.5% and 97%, and the specificity 100% and 99.4% for the SDA and LCx tests respectively. Similarly in female urine samples the respective figures were 77.3%, 86.4% and 100%, 100%; and in female endocervical swabs were 81.8%, 90.9% and 100%, 100%. In female patients where both urine and endocervical swab were submitted the results were 95.6%, 100% and 100%, 100% respectively.

In conclusion the SDA tests is less sensitive than the LCx particularly on female urine samples. The results confirm that approximately 10% of female infections are missed if endocervical swabs only are used, due to urethral only infections, similarly if urine samples only are used there is a loss in sensitivity by both tests due to the low concentration (or absence) of *C. trachomatis* in this sample.

1230 A review of acute hepatitis a in injecting drug misusers in Grampian

M. BEADSWORTH, P. MOLYNEAUX, A. CADWGAN, A.R. MACKENZIE, D. ROBSON, J.G. DOUGLAS and R.B.S. LAING
Infection Unit, Aberdeen Royal Infirmary

We report 6 cases of hepatitis A diagnosed in injecting drug misusers in Grampian, between October and December 2000.

All cases presented with symptoms and signs of acute hepatitis and serology confirmed acute hepatitis A. The method of transmission was not proven, but was presumed to be due to poor hygiene and/or sharing of needles and apparatus. There is the possibility of more severe illness due to acute hepatitis A in those who have chronic liver disease due to hepatitis B or C.

The ongoing epidemic of acute hepatitis B in Grampian related to injecting drug misusers has led to a large vaccination programme against hepatitis B. Hepatitis A immunisation should also be considered for this risk group, especially as some have already been infected with hepatitis C and others are at ongoing risk.

1245 Cytokine and nitric oxide responses to Japanese encephalitis virus infection in CSF and plasma

P.M. WINTER[1,2], N.M. DUNG[4], H.T. LOAN[4], R. KNEEN[3], L.T. THAO[4], D. HOUSE[3], J. FARRAR[3], N.J. WHITE[3], C.A. HART[2] and T. SOLOMON[1,2]
[1]Depts of Neurological Science, and [2]Medical Microbiology and Genitourinary Medicine, The University of Liverpool, Duncan Building, Daulby Street, Liverpool, L69 3GA, [3]Wellcome Trust Clinical Research Unit, [4]The Centre for Tropical Diseases Ho Chi Minh City, 190 Ben Ham Tu, Quan 5 Ho Chi Minh City

Japanese encephalitis (JE) is a CNS infection, which is caused by the *Japanese encephalitis virus* (JEV). JEV is a member of the genus *Flavivirus*, it is a single stranded positive sense RNA enveloped virus, which is spread by the mosquito *Culex tritaeniorhynchus*. JE is the most common cause of viral encephalitis worldwide, causing approximately 50,000 cases and 10,000 deaths per year. Most people in Asia are exposed during childhood, but only 1:25–1:1000 develop the clinical disease.

The aim of the work was to compare the cytokine and nitric oxide (NO) levels in CSF and plasma samples from 178 Vietnamese JE patients with varying disease outcomes, to try and identify specific cytokine responses to JE, and JE outcome. The cytokines assessed, included members involved in the T_H1 and T_H2 responses, as well as non-inflammatory and pro-inflammatory cytokines; IL-4, IL-6, IL-8, TNF-α, IFN-α, IFN-γ, RANTES, and also total NO levels. All were assayed using ELISA.

In summary the results showed raised levels for all cytokines and NO, except that of IFN-α, in both plasma and CSF. In the CSF the cytokines IL-6, IL-8, and IFN-γ showed an increasing level with worse clinical outcome, whereas NO showed the inverse association.

1300 Photoantimicrobial agents in blood product disinfection (Pathogen Inactivation Technology)

MARK WAINWRIGHT
Reader in Medicinal Chemistry, Centre for Forensic Science, University of Central Lancashire, Preston PR1 2HE

The use of photosensitizers as disinfecting agents in blood products is discussed. This is an emerging technology based on the designed selectivity of photosensitizers for microbes relative to blood components - erythrocytes, thrombocytes, plasma proteins etc. Problems so far encountered have centred on delivering selective inactivation, mainly of viral contaminants such as HIVand hepatitis, although photobactericidal and photoantiprotozoal action is of equal relevance. This problem is made more complex by the presence of e.g. intracellular pathogens which, by definition, require the passage of the photosensitizer to the cell interior, and also by the light absorption of endogenous pigments such as haems, particularly in erythrocyte concentrates. Both

of these factors limit the type of photosensitizer which may be employed. The combination of subject areas involved in such multidisciplinary research makes this a complex field which has yet to be recognised properly in the UK.

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0930 Quality and standards in Higher Education: the new QAA method for Academic Review

PETER MILTON
Director of Programme Review, The Quality Assurance Agency for Higher Education, Gloucester

The 1992 Further and Higher Education Act, and the subsequent identification by the (then) Secretary of State of the reasons for carrying out assessments (reviews) of the quality of educational provision, established the broad parameters within which the QAA has functioned. These were expanded, and to some extent superseded, by the Dearing Report of 1997, which set the agenda for much of the QAA's developmental work and brought scrutiny of academic standards into the process of review for the first time.

This presentation examines the means by which the Agency is taking the Dearing recommendations forward, in particular the means relevant to the operation of reviews at the level of the subject. It also outlines the motives underpinning the moves from a relatively inflexible method of subject review to a method which emphasises variation in process but leads to consistency of outcomes. Such variation manifests itself particularly in the reduction of burden of external review on institutions and their staff – the so-called 'lighter touch' frequently demanded by those involved in higher education. Academic review may thus be regarded as part way down the evolutionary path from rigorous and universal external scrutiny, in which all are subjected to the same process, to a process emphasising almost complete reliance on internal systems for quality assurance, perhaps merely sampled by an external body such as the QAA to ensure adherence.

Some discussion of the factors involved in 'lighter touch' is presented and the presentation goes on to examine the main differences between the review methods experienced hitherto and those developed for the period 2000-6. In discussing the 'headlines' of the new process, the presenter will speculate about the likely continuing evolution of review methods at the level of subject and the institution.

1015 Benchmarking academic standards - BSc Honours degree in chemistry

EDWARD ABEL
University of Exeter

The panel for Chemistry had a challenging remit in that they were the "first off the blocks" and there were no substantial precedents. This had both its pros and cons. Additionally, the work was being pursued at a time when QAA, and benchmarking in particular, were having a very mixed press.

The benchmarking of degrees is now building into a significant division of the Quality Assurance Agency's portfolio of academic standards; and in the future subject knowledge content and the teaching and assessment of degree courses will need to accommodate it.

It will be useful to review the make up and expertise of the chemistry benchmarking panel, and how it worked, consulted and eventually reported. The interface of a benchmarking document with the degree structure and knowledge content at various institutions is sensitive. The most difficult part of the chemistry panel's considerations concerned the `subject knowledge' content of the document.

Guidance on other aspects of the degree course such as aims, abilities and skills, assessment and performance are included.

Wide consultation of academic departments, commerce and industry, and professional bodies were made during the preparation of the chemistry document.

1120 QAA Trialing – a chemistry experience

ISOBEL WALKER
Dept of Chemistry, Heriot-Watt University, Edinburgh EH14 4AS

During 1998-99, Heriot-Watt University took part in trials of the QAA proposals for work related to academic standards (outcomes) in Chemistry. These involved preparation of
(i) programme specifications for typical Chemistry degree programmes and,
(ii)detailed responses to 6 specific questions related to learning outcomes.
The University was subsequently visited by three Assessors who met with selected groups of people.

This presentation will describe, briefly, the trialing procedures and give a "personal" view of the experience.

1155 An Award is Greater than the Sum of its Parts: a discussion of multidisciplinary programmes

JENNIFER MOON
University of Exeter

A flood of Quality Assurance Agency papers has poured into higher education in the last year; the position paper on the National Qualifications Framework (NQF) is out and QAA benchmark statements are written for over half of the subject groups. Yet most of this literature is directed towards the type of higher education programme that only around a third of students applies for – the single honours programme. Over two thirds of students, this academic year, applied to follow programmes that represent more than one discipline. The range of multidisciplinary programmes is broad, from joint honours to the truly multidisciplinary programme. While there are relatively few programmes at the latter extreme, the issue of coherence in all of these programmes is important. Indeed it is often a topic of discussion even within the single honours curriculum.

In this session we will consider the words of two QAA reports (NQF and that from the Multidisciplinary Advisory Group), that an award is greater than the sum of its parts. Should an award represent something greater than the sum of its contributory modules – or is it all right that it amounts only to the sum of learning that a student will have experienced from a range of separate modules? We will argue good reasons for the former, making suggestions as to how the quality of coherence can be brought into a programme without stepping outside its modular structure.

1350 Benchmarking bioscience with particular reference to microbiology

PAUL F. BRAIN
School of Biological Sciences, University of Wales Swansea, Swansea SA2 8PP

Benchmarking (or specifying the general characteristics of degree schemes for the presumed benefit of students, parents and potential employers) is an exercise entrusted to the QAA. It largely arose as a recommendation of the NCIHE (Dearing) report on standards and a decision was made to entrust this activity (initially in relation to the 'honours' degree) to small expert groups from the academic community.

'Biosciences' is an extremely diverse multidisciplinary area often with major interactions with chemistry, geography, geology, oceanography, physics, mathematics and IT. The area thus encompasses a range of sub-disciplines, all of which involve the study of diverse and variable living organisms and systems often in changing environments. In addition to wide-ranging degrees such as Biology, Biological Sciences and Life Sciences, there are sub-disciplines within this area that focus on particular groups of organisms (e.g. Microbiology, Mycology and Virology). Other degrees

emphasise specific technologies, approaches or systems (e.g., Biochemistry, Cell Biology, Genetics, Immunology, Infection Biology, Nutrition and Pharmacology). Another group is concerned with the environments that living organisms inhabit (e.g. Environmental Biogeochemistry). Some sub-disciplines are directed towards particular applications (e.g. Brewing and Distilling, Nutritional Biochemistry, Toxicology and Wine Studies).

The panel would appreciate views from the community on a variety of issues (see below).

Is there any non-trivial area of biology that all Bioscience graduates must know?

Bioscience degrees frequently involve studies at a variety of levels (from the molecular, through cells, tissues, organs and whole organisms and, in some cases, extending to populations or communities). It seems important that all students have some appreciation of these levels. This may be a lesser concern for Microbiologists but do they have a view?

Developments in molecular and structural biology have provided recent (but incompletely utilised) opportunities to unify the disparate areas. Have Microbiologists any specific ideas on how best to do this?

Most Bioscience subjects exist in an environment of current hypothesis rather than certainty. This means that many students must be given training in comparing the merits of alternative hypotheses and guided in terms of how to construct experiments or to make observations to challenge them. Does this apply to Microbiology?

The Biosciences includes areas (especially molecular approaches) in which rapid developments are evident and where new knowledge and technologies are swiftly spread through the subject. This means that there is an increasing requirement to prepare its graduates carefully for continuing their self education and development after graduation to maintain the currency of their degrees in rapidly changing areas.

Is this a concern in Microbiology?

Biosciences are essentially practical and experimental subjects. Consequently, appropriate opportunities to do some 'hands on' investigations (e.g. laboratory studies for most groups) are seen by many practitioners as integral to any scheme of study in this area. These, of course, can be supplemented by modern IT- assisted approaches but are practical essential and how does one achieve balance? The appreciation of hypothesis formation and testing is often developed by project work in the various sub-disciplines. Should a project be an integral part of any Biosciences degree?

The Biosciences are subjects that combine scientific rigour with an acceptance of diversity and variability, thus providing an ideal training for the complexities of life. Many of the degree schemes provide general skills and competencies suitable for the world of work in which the focus is not biology. Studies in the Biosciences encourage an understanding of multidisciplinarity, an enquiring attitude and an appreciation of complexity. They require development of competencies in team and individual working, numeracy (often including IT and statistics) as well as oral and written proficiencies. Are all these required by Microbiologists?

After a period of slow change, the Biosciences (during the latter half of the 20th century) have advanced to the forefront of new technologies. Biology now has a major impact on most aspects of human activity. Biology is currently at the frontier of public awareness. The view has been expressed that the subject needs to respond to its high public profile by providing students with training on how to deal with ethical, moral and exploitation issues. Do Microbiologists subscribe to the view and, if so, how do they do it?

member's perspective

HELEN O'SULLIVAN
Dept of Human and Biological Science, Liverpool Hope University College, Hope Park, Liverpool L16 9JD

"Biosciences" is arguably the most diverse science and attracts many students over a range of exciting academic disciplines. The current importance of these subjects to society is reflected in any sample of newspapers or current affairs programmes. The task of the Benchmarking Panel was therefore onerous. How to come up with a set of defining principles for biosciences, a list of knowledge, understanding and skills that all students graduating in any of the biosciences subjects should demonstrate and guidelines on assessment and standards?

The first meeting of the Bioscience Benchmarking Panel was in September 2000. One of the first items for discussion was whether we could agree on any defining principles in Bioscience. This proved to restricted to statements so superficial as to be thought by the group to be meaningless. The current position is that the subject can be best defined by a series of skills and attributes rather than specific content.

How will this impact on teaching microbiology? So far, it seems that microbiologists will still be able to choose their curriculum to reflect the needs of the students, the facilities available and the interests of the staff. However, we will all be much more accountable for describing the ways in which these curricular are delivered. Levels and standards will need to be clearly articulated and we will need to find ways to clearly demonstrate that outcomes are at the appropriate level. There is still an issue about how the level of a skill or attribute is clearly defined. For example, most Bioscience programmes would contain an outcome along the lines of *design and carry out an experiment/investigation*. However, this outcome is appropriate to a range of awards from GCSE to PhD. One of the aims of Benchmarking will be to give us a shared vocabulary with which to describe these levels.

1505 The ILT-supporting quality learning & teaching in microbiology?

LIZ SOCKETT
Genetics Division, School of Clinical Laboratory Sciences, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH

The Institute for Learning & Teaching in Higher Education (ILT) has been running for about 18 months. It was set up, following recommendations in the Dearing Report, to "support excellence in learning & teaching" in HE. Initially it was also suggested that ILT membership might be taken into account in future QAA reviews of teaching standards in universities. ILT Membership for new academics is achieved by completion of an approved postgraduate course in academic practice. Currently (until September 2001) for experienced academics, membership can be awarded subject to satisfactory submission of an application form, a portfolio of experience and a c.v.. This talk is given from the perspective of an ordinary ILT member who is a microbiology academic.

It will address issues including: How does ILT support science academics? Is the ILT journal a source of useful educational practices? What are ILT meetings like? Should microbiology academics join before the Sept 2001 deadline? What is the application process like? What are future plans for ILT and how will it relate to QAA processes in HE?

1430 Benchmarking the microbiologist: a panel

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0910 Microbe-pollutant and microbe-microbe interactions involved in accessing and exploiting hydrophobic pollutants

K.N. TIMMIS
Division of Microbiology, GBF-National Research Centre for Biotechnology, Braunschweig, Germany

Many industrial xenobiotic organic compounds are toxic for higher organisms, persist in the environment, and thus are important pollutants. On the other hand, some are catabolised by microorganisms, which thereby play a key role in pollutant elimination from the environment. Hydrophobic xenobiotics, particularly complex mixtures of congeners and compounds, constitute both nutritional opportunities and, as a result of their toxicity and solvent activity on cell membranes, physiological and metabolic challenges for terrestrial, subsurface and aquatic microorganisms. The challenge for a better exploitation of microbial metabolic potential to eliminate pollutants from the environment is to gain an understanding of how microbial communities that tolerate and exploit such compounds develop, and how the member populations interact with the pollutants and with one another, to extract maximum nutritional benefit. In this presentation, I shall report on experiments with field samples and experimental models that document critical interactions between populations of diverse microbes, between the microbes and the pollutant, and between the microbes and soil matrix material. The results obtained, together with those of others, suggest non-native surface structures of interacting pollutant and microbes within metabolically-structured cooperative biofilm communities that represent functional units which assure maximum access to and utilisation of novel toxic substrates with minimum sustainment of cellular damage.

0955 Pathways to bioremediation of nitroaromatic compounds

JIM SPAIN
US Air Force Research Laboratory, 139 Barnes Dr., Tyndall AFB, FL, 32403-5323, USA

Nitroaromatic compounds are widely distributed in the environment because of their use in explosives, dyes, plastics, and detergents. They are truly xenobiotic and natural analogues are uncommon. They have only been released in substantial amounts during the past century, so bacteria able to degrade them have probably evolved the capability only recently. Bacteria able to attack most of the simple nitroaromatic compounds have been isolated from contaminated sites, which indicates that some biodegradation is taking place in situ. Catabolic pathways used by bacteria to derive carbon and energy from nitroaromatic compounds are fairly well established. Several questions remain regarding the regulation and evolutionary origin of the catabolic pathways, inhibition and misrouting during degradation of multiple contaminants, and strategies for practical applications in bioremediation. Strategies for biodegradation of dinitrotoluenes have led to in situ field applications at contaminated sites where conditions are appropriate. The two predominant isomers are not degraded simultaneously so treatment systems in the field must be designed to allow for sequential processes. Recently discovered strains able to degrade both isomers could overcome the problems associated with degradation of mixtures.

Nitrobenzene can be degraded via several distinct pathways. The partially reductive pathway seems to be predominant in contaminated subsurface sites. The initial reactions in the pathway involve an unusual membrane-bound mutase that catalyzes the conversion of hydroxylaminobenzene to aminophenol. It is clear that the reaction involves an intramolecular transfer of the hydroxyl group, but the mechanism of the reaction and the evolutionary origin of the enzyme are unknown. The subsequent steps in the pathway are analogous to those of the catechol meta cleavage pathway, but the genes are only distantly related.

1110 Microbial metal transformations: a solution to a problem?

GEOFFREY M. GADD
Division of Environmental and Applied Biology, Biological Sciences Institute, School of Life Sciences, University of Dundee, Dundee DD1 4HN

Microorganisms are intimately involved in the biogeochemical cycling of metals and metalloids. Certain microbial processes solubilize metals thereby increasing their mobility, which may increase bioavailability and potential toxicity, whereas other processes result in immobilization and reduce bioavailability. Metal mobilization can arise from autotrophic and heterotrophic leaching, chelation by metabolites and siderophores, and methylation which can result in volatilization. Immobilization can result from sorption to cell components or exopolymers, transport and intracellular sequestration or precipitation as organic and inorganic compounds, e.g. oxalates in fungi, and sulphides in sulphate-reducing bacteria. In addition, biologically-mediated reduction of higher-valency species may effect mobilization, e.g Mn(IV) to Mn(II), or immobilization, e.g. Cr(VI) to Cr(III). In the context of bioremediation, solubilization of metal contaminants provides a means of removal from solid matrices such as soils, sediments, dumps and other solid industrial wastes. Alternatively, immobilization processes may enable metals to be transformed *in situ* and are particularly applicable to removing metals from aqueous solution.

This contribution will detail selected microbiological processes which are of significance in determining metal mobility and which have actual or potential applications in bioremediation of metal pollution. These include autotrophic and heterotrophic leaching mechanisms, reductive precipitation, sulphate reduction and metal sulphide precipitation. A particular example is the application of microbially-catalyzed reactions which occur in the natural sulphur cycle. In one integrated microbial process for the bioremediation of soil contaminated with toxic metals, sulphur-oxidizing bacteria were used to leach metals from contaminated soils by breakdown of minerals and liberation of acid-labile forms. The combination of bioleaching followed by separate bioprecipitation of leached metals by SRB, was effective in removing and concentrating a range of metals including Zn, Cu and Cd from metal-contaminated soil. Sulphate-reducing bacterial biofilm reactors may offer a means of process intensification and these systems entrap or precipitate metals, e.g. Cu and Cd, at the biofilm surface. Mixed SRB cultures were found to be more effective than pure cultures for metal removal, which was enhanced by the production of exopolymers. Where reduction of a metal to a lower redox state occurs, mobility may be reduced. Such processes may accompany other indirect reductive metal precipitation mechanisms, e.g. in sulphate-reducing systems where reduction of Cr(VI) can also result from indirect reduction by Fe^{2+} and the produced sulphide. However, we have found that chromate reduction by mixed culture SRB biofilms is predominantly a biological process.

The microbial production of organic acids, under the general heading of “heterotrophic leaching”, is of developing interest in pollution treatment and metal recovery. In most fungi, leaching is mediated by the production of organic acids which provide a source of protons and complexing organic acid anions. This mechanism provides a means of metal solubilization from insoluble metal compounds

including solid wastes and minerals. Heterotrophic solubilization can also have consequences for other remedial treatments for contaminated soils. Pyromorphite ($Pb_5(PO_4)_3Cl$) is a stable lead mineral and can form in urban and industrially-contaminated soils. Such insolubility reduces lead bioavailability and the formation of pyromorphite has been suggested as a remediation technique for lead-contaminated land, if necessary by means of phosphate addition. However, pyromorphite can be solubilized by phosphate-solubilizing fungi, e.g. *Aspergillus niger*, and plants grown with pyromorphite as a sole phosphorus source accumulate both P and Pb. Further, during the fungal transformation of pyromorphite, the biogenic production of lead oxalate dihydrate was observed for the first time. This study emphasises the importance of considering microbial processes in developing remediation techniques for metal-contaminated soils. Such mechanisms of lead solubilization or immobilization as lead oxalate may have consequences for metal mobility and transfer between environmental compartments and organisms.
GMG gratefully acknowledges financial support from the Biotechnology and Biological Sciences Research Council.

1155 Bio-indicators to assess impacts of heavy metals in land-applied sewage sludge

JACQUI HORSWELL, TOM SPEIR and ANDREW VAN SCHAIK
ESR Ltd, Kenepuru Science centre, Kenepuru Drive, PO Box 50-348, Porirua, New Zealand

Application of sewage sludge to land provides a convenient method for disposal of a waste product, and has the beneficial aspect of adding plant nutrients and organic matter to the soil. However, sewage sludge also contains potentially toxic elements, such as heavy metals, and there is concern about the long-term effects of these metals on the soil. Adverse effects of heavy metals on soil biological and biochemical properties are well documented. Such studies lie at the root of current research efforts to utilise the metal sensitivity of some of these soil properties to monitor soil pollution and to derive effects-based soil pollutant limits. Most of the research has involved short-term laboratory investigations, but it is recognised that the responses obtained in the laboratory may bear little relation to those seen in the field. However, field studies often suffer from a lack of a suitable control, may not display a range of soil concentrations, and are usually contaminated by several metals. In this project, we have attempted to overcome the shortcomings of field experiments by spiking sewage sludge with single heavy metals (Cu, Zn and Ni) and amending plots over a range of metal concentrations. We have measured effects of heavy metals on: basal respiration, microbial biomass C, sulphatase and phosphatase enzyme activities, MPN of *Rhizobium* and response of a biosensor based on *Rhizobium leguminosarum* biovar. t*rifolii* (Rhizotox-C). Preliminary results from the first two years of sampling showed no effect due to spiked sludge amendment on microbial biomass C or phosphatase activity. Toxic metal effects were seen in the highest metal plots for sulphatase activity, and basal respiration, this was especially apparent for the highest Zn plot (nominally 400 mg/kg Zn). Inhibitory effects on Rhizotox-C, and on MPN of *Rhizobium* were also demonstrated in the highest metal plots.

1210 Fungal solubilization of insoluble metal phosphates under nutritionally-heterogeneous conditions

HELEN JACOBS[1], GRAEME BOSWELL[2], KARL RITZ[3], GEOFFREY M. GADD[1] and FORDYCE A. DAVIDSON[2]
[1]Division of Environmental and Applied Biology, School of Life Sciences, [2]Dept of Mathematics University of Dundee, Dundee, DD1 4HN, [3]Soil-Plant Dynamics Group, Scottish Crop Research Institute, Invergowrie DD2 5DA

The ability of fungi to solubilize insoluble metal phosphates is an important process for both plant and microbial nutrition. However, the spatial distribution of nutrients in terrestrial ecosystems is generally heterogeneous due to environmental factors such as soil type and structure. The objectives of this study were to determine the ability of *Rhizoctonia solani* to solubilize metal phosphates under nutritionally-heterogeneous conditions. *R. solani* was able to solubilize a number of metal phosphates including $Ca_3(PO_4)_2$, $Sr_x(PO_4)_x$, $Mn_3(PO_4)_2$, $Co_3(PO_4)_2.8H_2O$ and $Zn_3(PO_4)_2.H_2O$. The latter two compounds were only solubilized by *R. solani* at low concentrations (≡ 5mM) and these metal phosphates inhibited growth at a higher concentration (≡ 15mM). Oxalate crystals were only produced in $Ca_3(PO_4)_2$- and $Sr_x(PO_4)_x$-amended medium. The rate of solubilization (expressed as colony extension rate: rate of extension of the clear zone) was twice as high for *R. solani* grown at 15°C than when grown at 30°C, even though the growth rate was reduced by more than half at the lower temperature. In further experiments, tessellations of agar tiles, with and without the inclusion of glucose and metal phosphates, were used to study metal phosphate solubilization under nutritionally-heterogeneous conditions. Since the production of organic acids is dependent on the carbon source, translocation of carbon may be determined if solubilization of the metal phosphate occurs. The applicability of this, and related techniques, for studying metal phosphate solubilization in relation to heterogeneous distribution of nutrients will be discussed, as well as physiological and mathematical interpretation of growth and solubilization kinetics.
Financial support is gratefully acknowledged from the Biotechnology and Biological Sciences Research Council (94/MAF12243).

1345 Implications of soil-contaminant contact time on the biodegradation of organic chemicals in soils

KIRK T. SEMPLE[1], JOANNA D. STOKES[1], PAULA CLASPER and BRIAN J. REID[2]
[1]Dept of Environmental Science, Lancaster University, Lancaster, [2]School of Environmental Sciences, University of East Anglia, Norwich

Hydrophobic organic contaminants (HOCs), such as polycyclic aromatic hydrocarbons (PAHs), enter soils via atmospheric deposition and industrial and agricultural activities. These compounds can associate with/partition strongly onto soils. It has been observed that as soil-HOC contact time increases, HOC extractability decreases. Similarly, decreases in bioavailability with increased soil-HOC contact time have been described in bacterial, earthworm and other organism studies. This phenomenon has been termed “ageing”. Traditionally, assessment of contaminated land has been concerned with the determination of total organic contaminant concentrations in soils, using exhaustive extraction techniques. However, in light of the increasing body of knowledge relating to temporal decreases in contaminant availability in soils, such methods may have little relevance to the amount of contaminant, which may pose an ecological risk or be available for degradation, i.e. the bioavailable fraction. An extraction method using cyclodextrins has been developed to determine contaminant availability to microbes in soils. In recent work, PAH extractability using dichloromethane and butanol as well as cyclodextrin extraction was compared to HOC degradability. In all cases, the correlations between extractability and degradability were high. However, only the cyclodextrin extractions gave a 1 to 1 comparison between PAH extractability and biodegradability in soil. Recently, we have investigated the feasibility of using the cyclodextrin extraction technique on a PAH-contaminated soil from a disused coke plant to determine the putative bioavailability of the PAHs present in the soil. As before, a strong 1 to 1 relationship was found between the mass extracted and degraded for 16 PAHs in the soil. It is anticipated that by using cyclodextrin extraction procedures, assessments for the success of bioremediation as well as risk, can be made in soils contaminated with HOCs.

1430 Shedding light on metal availability to microbes in soil: *lux* biosensors applied to the solid and solution phase

KEN KILLHAM, SARA PRESTON and GRAEME PATON
Dept Plant & Soil Science, University of Aberdeen, Cruickshank Building, St. Machar Drive, Aberdeen AB24 3UU

The bioavailability of metals to microbes represents both a key control of the productivity of the plant-soil system and a critical determinant in the bioremediation of many soils contaminated by urban and industrial activities. Despite the importance of metal toxicity in soils, there are few reliable methods to rapidly assess metal bioavailability to soil microbes.

Microbial biosensor technology now offers a powerful way forward to not only assess the availability of metals, but to also investigate the factors controlling availability and to design/monitor strategies for the bioremediation of soils where the activity of the bioremediating microbes is constrained by metal toxicity. This biosensor technology is based on the use of luminescence (*lux*) reporter gene systems incorporated into the chromosome of indigenous soil bacteria such as species of *Pseudomonas, Burkholderia* and *Rhizobium*, with contrasting properties in terms of susceptibility to metals, ecophysiology and predominant microhabitat.

Microbial biosensor response data are presented for three case studies. The first is a distillery in Speyside where adjacent soils have received copper contaminated wastes. The second is a petrochemical plant where metals have been used and, as a result, the soil is co-contaminated with chlorinated/non-chlorinated hydrocarbons and lead/zinc. The third is a group of sites which have received metal-contaminated sewage sludge with a range of metal concentrations. The biosensor data demonstrate the dynamics of metal availability to soil microbes at these sites and the importance of the degree of buffering/protection offered by the different soils. Data from solution and solid phase biosensor assays are then presented to demonstrate some spatial aspects of metal pollutant toxicity to microbes in soils. Finally, data from radiolabelling experiments are presented to assess the degree to which heavy metal toxicity is a constraint to microbial degradation of organic pollutants and to assess the correlation between biosensor prediction and actual mineralisation of ^{14}C organopollutants by the indigenous soil microbial population.

Further questions which can be addressed using biosensor-based assessment of metal bioavailability to soil microbes are proposed in conclusion, to improve our understanding of this under-researched aspect of microbial ecology and to ensure that we better manage metal contaminated soils in the future.

1545 Influence of the rhizosphere on biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4-dichlorophenol (2,4-DCP) in soil

LIZ J. SHAW and RICHARD G. BURNS
Research School of Biosciences, University of Kent at Canterbury, Kent CT2 7NJ

Studies have shown that the rate of biodegradation of organic xenobiotic compounds may be enhanced in the presence of the rhizosphere when compared to biodegradation in non-rhizosphere soil. However, the specific mechanisms by which plant roots influence microorganisms to bring about increases in biodegradation rate have not been thoroughly investigated. One hypothesis is that compounds in root exudates may fortuitously select or stimulate specific microbial groups with biodegradative properties. In addition to direct effects on the number, activity and composition of microbial communities, the influence of plant roots on the physico-chemical properties of the rhizosphere environment may also indirectly effect xenobiotic fate. Specifically, changes in the pH and quality and quantity of the soil organic carbon compounds in rhizosphere soil may alter the bioavailability of the xenobiotic. Recent research on the direct and indirect influences of the *Lolium perenne* and *Trifolium repens* on the biodegradation of 2,4-D and 2,4-DCP in the rhizosphere will be discussed and the potential for rhizoremediation of contaminated soils evaluated.

1600 Bacterial plasmids as allies in the biodegradation of agrochemicals

CHRISTOPHER W. SMEJKAL[1], TATIANA VALLAEYS[2], SARA K. BURTON[1] and HILARY M. LAPPIN-SCOTT[1]
[1]University of Exeter, School of Biological Sciences, Prince of Wales Road, Exeter EX4 4PS, [2]Lab Microbiology, Radioactive Waste and Clean-up Division, SCK/CEN, Boeretang 200, B-2400-MOL-Belgium

Phenoxyalkanoic acids constitute a major group of organic herbicides. Among them, 2,4-D and mecoprop have been widely used to destroy broad-leaved weeds in a number of countries since World War II. However, while 2,4-D degradation has been well studied both at the biochemical and the genetic level, very few studies have focused on bacteria with the ability to degrade mecoprop. Based on the knowledge reported on 2,4-D, this study focuses on establishing whether there is a genetic link between three mecoprop degrading bacteria; *Alcaligenes denitrificans*, *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 and the 2,4-D degrading *Variovorax paradoxus* TV1. All three mecoprop degrading strains, isolated from British agricultural soils harbour large catabolic plasmids. DNA hybridization experiments and phylogenetic analysis of the *tfd*A, *tfd*B and *tfd*C gene families demonstrated that *Alcaligenes* sp. CS1 and *Ralstonia* sp. CS2 carry genes presenting over 60% sequence similarity to *tfd*A, *tfd*B and *tfd*C genes of TV1, while *A. denitrificans* plasmid carried fragments with 60% or more sequence similarity to *tfd*A and *tfd*C only. Loss of the catabolic plasmids in all strains resulted in loss of degradation. A study of TFDA enzyme activity suggests that a broad substrate range enzyme is involved in the first step in both pathways and is not dependent on the *tfdA* gene type. This work thus demonstrates a genetic link between the genes involved in the degradation of the two related compounds; 2,4-D and mecoprop.

1615 Aromatic compound degradation by novel acidophilic bacteria with potential use for the treatment of waste-water containing organic and inorganic pollutants

KEVIN B. HALLBERG, PETER A. WILLIAMS and D. BARRIE JOHNSON
School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UW

In the fine chemical and metal industries, large quantities of waste waters are generated that contain both organic and inorganic pollutants. The organic pollutants are often aromatic compounds, while the inorganic pollutants consist of metals that are used as catalysts. In order for many of the metal catalysts to be maintained in solution, these waste waters are generally acidic. Currently, such wastes are treated by chemical means, or at minimum with an adjustment of the pH before biological treatment. While there has been one report describing the transfer of a plasmid encoding for the degradation of phenol to an acidophilic bacterium, there has been no report of any acidophile with the capacity to degrade aromatic compounds. We have isolated acidophilic bacteria with the capability to use aromatic compounds as sole carbon and energy compounds by enrichment. One group of acidophiles exhibited an ability to grow with high concentrations (mM range) of various aromatic acids. These

microbes are phylogenetically related to known acidophilic bacteria of the genus *Acidocella*. One of the isolates, called WJB-3, is able to grow on a wide range of aromatic compounds, including many recalcitrant aromatics. This acidophile was successfully used in a column bioreactor to treat a model acidic waste water containing both organic and inorganic pollutants.

1630 Rhizosphere-microbe-pollutant interactions

S.P. McGRATH, F.J. ZHAO and E. LOMBI
Agriculture and Environment Division, IACR-Rothamsted, Harpenden, Herts AL5 2JQ

Metal inputs still exceed the outputs in the majority of European soils, so metals are accumulating in topsoils. As a consequence of the need to recycle organic wastes, it seems likely that the inputs of toxic metals will increase further. The residence time of metals in soil is of the order of thousands of years, so novel technological approaches are required to remove excess toxic metals.

One branch of bioremediation, called phytoextraction, aims to use plants to extract and remove toxic metals from soils, and offers the benefit of being low cost, in situ and environmental sustainable. Three phytoextraction approaches are being developed, which aim to extract toxic metals from soil: 1) use of hyperaccumulator plants with exceptional metal-accumulating capacity (natural phytoextraction), 2) use of high biomass crops which are only induced to take up large amounts of metals when the mobility of metals in soil is enhanced with chemical treatments (chemically-assisted phytoextraction), and 3) phytovolatilization.

In the case of hyperaccumulators, acidification of the rhizosphere does not appear to be involved in the enhanced uptake observed. It is not yet known whether hyperaccumulating plants exude specific compounds to help mobilise metals in soils, and the rhizosphere processes that limit phytoextraction have not yet been identified and modelled. Chemically-assisted phytoextraction involves adding compounds that increase the metal concentrations in the rhizosphere, but application of other substances are probably also necessary to break down the barriers to metal uptake by the plants. It seems unlikely that soil microbes such as mycorrhiza have a large role to play in current methods that extract metals into plants, but in the case of soils polluted with persistent organic compounds they are of paramount importance. In the case of phytovolatilization, soil microorganisms and plants appear to be involved.

FRIDAY 30 MARCH 2001

0900 Risk assessment of contaminated soils using microbial biosensors

SOFIA SOUSA[1], KEN KILLHAM[1,2] and GRAEME I. PATON[1,2]
[1]Remedios Limited, 3 Rubislaw Terrace, Aberdeen AB10 1XE - Tel 01224 415955, Fax 01224 415966; Email: **Error! Bookmark not defined.**, [2]Dept of Plant and Soil Science, Cruickshank Building, University of Aberdeen AB24 3UU - Tel 01224 272692, Fax 01224 272703; Email: **Error! Bookmark not defined.** and **Error! Bookmark not defined.**

As legislation becomes more directed towards contaminated land, there is a need for owners and developers to have a rigorous understanding of the extent and nature of contaminants in their sites. Detailed knowledge is paramount as it enables identification of *hot-spots* and effective cost prediction of remediation/ bioremediation protocols.

Environmental toxicity screening requires rapid, reliable and sensitive monitoring procedures. *Lux*- based bacterial assays have been developed for rapid toxicity testing of industrial effluents, contaminated groundwater and toxicity monitoring of sites undergoing bioremediation. Expression of the target genes in the *lux* biosensors is constitutive and linked to bacterial metabolic activity, and therefore bioluminescence decreases in the presence of toxic pollutants.

Contaminated sites were assessed using both conventional analytical techniques and the bioluminescence-based biosensors. A herringbone sampling strategy was defined and the intensity of sampling increased around areas where pollution incidents were deemed to have been most significant.

Chemical analysis demonstrated that there were several significant elevated levels of contaminants at the sites. The biosensor analysis was able to identify and quantify the risk posed by these contaminants. The results were represented spatially using a "Toxmap™" in order to facilitate the visualisation of the *hot-spots* of pollutants at the site and the interpretation of the results.

The biological techniques correlated closely with the metal and organic pollutant loads and with each other. The biosensor assay was found to be the most rapid and able to identify risks associated with contaminant toxicity.

0945 Molecular probes: a measure of cyanide bioremediation by fungi?

MICHELLE BARCLAY[1, 2], MARK J. BAILEY[1], CHRIS J. KNOWLES[2] and IAN P. THOMPSON[1]
Oxford Centre for Environmental Biotechnology at [1]NERC CEH-Oxford, Mansfield Rd, Oxford, OX1 3SR, [2]Dept of Engineering Sciences, University of Oxford, Parks Road, Oxford OX1 3PJ

PCR based molecular probes can be used to determine degradative potential in an environment by detection of degradative loci in relation to gene expression using mRNA RT-PCR, followed by examination of environmental factors that may limit gene expression. In this study cyanide degradation by the fungal cyanide hydratase gene (*chy*) is employed as the model system to investigate the potential of gene probes for *chy* expression in *Fusarium* spp. in order to optimise cyanide degradation *in situ*. *Chy* gene presence was confirmed by a 600 bp PCR product containing two 50bp introns. In conjunction with the 500bp amplicon from mRNA this provided an effective measure of RT-PCR fidelity.

Shake flask studies with *Fusarium* spp. pure cultures revealed that *chy* is induced by free cyanide (KCN) and metal complexed cyanide ($K_2Ni(CN)_4$) and is detected by RT-PCR 20-40 minutes after exposure to cyanide. Furthermore, expression was detected in soil spiked with both cyanide and *Fusarium* species.

Based on sequence analysis of the 600 bp region of the *chy* gene from a number of *Fusarium* isolates, enough variation was available to discriminate to the species level mixed cultures using DGGE or tRFLP. This information is sufficient to determine genetic cyanide-degradative potential and expression in a polluted environment. Combined with knowledge of limiting environmental factors, this enables the application of measures to increase gene expression and the identification of individual cyanide degrading species carrying out degradative activity in the environment.

1100 Natural attenuation of hydrocarbons in drill cutting piles: fact or artefact?

REBEKKA R.E. ARTZ[1,2], K. KILLHAM[1], JAMES I. PROSSER[2] and GRAEME I. PATON[1]
[1]Dept of Plant and Soil Science, University of Aberdeen, St Machar Drive, Aberdeen AB24 3UU, [2]Dept of Molecular and Cell Biology, Institute of Medical Science, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD

Oil production in the North Sea sector pre-1990 used predominantly oil-based drilling muds. The cuttings arising from drilling with these muds still contained significant amounts of residual muds and associated hydrocarbons despite cleaning prior to disposal onto the sea bed. The cuttings mounds building up during the drilling process cause several problems to the microbial and eukaryotic populations in the sediment due to physical smothering and toxic effects of the hydrocarbons or other constituents of the muds. Although a decrease in hydrocarbon levels has been

observed over a period of 10 years, to date it has been difficult to distinguish whether this 'decrease' is due to natural attenuation or whether it is an artefact of the sampling methods used. This study aimed to answer this question by determining whether the biological potential for hydrocarbon-degradation was in place. Several aerobic and anaerobic bacterial isolates capable of degradation of the main constituents of oil-based drilling muds were retrieved from drill cuttings material from the North West Hutton platform. These isolates were shown to be active under similar metal and hydrocarbon loads, as well as at the *in situ* temperature. Chemical biomass determination methods and molecular techniques (DGGE) indicate the presence of a highly active community with a population structure different to the surrounding clean sediment. Current work focuses on the determination of degradation rates and the involvement of functional bacterial groups in the degradation process.

1115 Microbial ecology and natural attenuation in a contaminated sandstone aquifer

ANNE TUCKER[1], TERRY McGENITY[1], ALWYN HART[2], ANDREW BALL[1] and KENNETH TIMMIS[1]
[1]Dept of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, [2]Environment Agency, Olton Court, 10 Warwick Road, Olton, Solihull, West Midlands B92 7HX

The microbial diversity of a contaminated aquifer was surveyed, with the aim of ascertaining the effects of contaminant concentration and other physico-chemical parameters on the structure of the microbial communities, and studying factors, which may influence the degradation of contaminants in situ. The aquifer is situated in a Triassic sandstone formation below a large chemical manufacturing plant in the UK, and is the focus of a research programme for natural attenuation (SIREN) involving the Environment Agency, AEA Technology Environment, and Shell Global Solutions. The contaminants include BTEX (benzene, toluene, ethylbenzene, xylene), naphthalene, styrene and chlorinated aliphatic hydrocarbons. Concentrations vary considerably, for example from 0.12 to 460 mg l^{-1} for benzene. Several strains have been isolated from the groundwater and, on the basis of partial sequencing of 16S rDNA, assigned to the following genera: *Pseudomonas* (four strains), *Acinetobacter* (three strains), *Microbacterium, Rhodococcus* and *Xanthobacter*. Total community DNA was extracted from groundwater samples taken from different wells, and T-RFLP (terminal restriction fragment length polymorphism) analysis used to generate community profiles independent of culture techniques. These profiles will be correlated with groundwater analyses carried out as part of the SIREN programme. Rapid and complete benzene degradation was observed in aerobic enrichment cultures (80 mg l^{-1} in 20 days or less) from two out of the three wells initially sampled. In cultures from the third well (pH 12.5), benzene degradation was highly inhibited. Benzene is one of the most persistent contaminants on the site and will subsequently be used in both aerobic and anaerobic cultures to enrich the benzene-degrading communities. T-RFLP will be used to examine the relative importance of the benzene-degrading consortia within the total bacterial community. Further experiments will test the factors which may inhibit or influence benzene degradation *in situ*.

1130 Functional and temporal changes in the microbial community during bioremediation in an *ex situ* biopile

SARAH CASSIDY, KENNETH BRUCE, ROLF HENKLER and JEREMY MASON
Division of Life Sciences, Kings College London, Franklin-Wilkins Building, 150 Stamford Street, London SE1 9NN

The *ex situ* remediation of benzene, toluene, ethylbenzene and xylene (BTEX) contaminated sediment from a paint manufacturing site was the focus of this study. Changes in biodegradative activity were linked to spatial and temporal changes in the microbial community. Biodegradative activity of the microbial community associated with the *ex situ* remediation of a 5000 m^3 biopile was studied over a 130 day period. Bioremediation potential was determined through radiolabelled pollutant mineralisation and selective microbiological counts. Rates of ^{14}C toluene mineralisation showed an initial increase in activity that was maintained until 96 days, after which the availability of hydrocarbon substrate probably became a limiting factor. Numbers of heterotrophic bacteria decreased during the treatment, however toluene degrading bacteria were seen to increase. DNA was extracted from soil samples taken at intervals and 16 S rRNA encoding genes amplified by Polymerase Chain Reaction. Equal length PCR products were resolved using Denaturing Gradient Gel Electrophoresis (DGGE) to provide a fingerprint of the microbial diversity within the biopile. Biodiversity, reflected in the numbers of bands detected, was initially low. A greater number of bands were present in material from the 29 and 57 day sampling points, indicating a greater level of diversity and corresponding with an increase in degradative activity. By 96 and 130 days the banding profiles were highly reproducible and indicated that the microbial community had returned to a more stable level of diversity concurrent with low mineralisation rates and the detection of low levels of BTEX contaminants. In this presentation this data will be discussed in relation to the restoration and return of biopile material to the excavation site where the soil may be challenged by reinfiltrating contaminated groundwater.

1145 Bioremediation of contaminated soil in biopiles

M.M. LAINE and K.S. JØRGENSEN
Research Laboratory, Finnish Environment Institute, POB 140, FIN-00251 Helsinki, Finland

Composting of chlorophenol-contaminated soil was performed first in small field scale (4 x 13 m^3 of soil) and then in large scale (520 m^3). The aim was to test the applicability of using straw compost (phase I mushroom compost), which was adapted for chlorophenol degradation, as inoculum in comparison to addition of bark chips and nutrients to contaminated soil. The starting concentrations of chlorophenols were from 800 to 900 mg (kg dry weight)$^{-1}$ or from 30 to 40 mg (kg dry weight)$^{-1}$. In all cases the chlorophenol degradation was more than 90% during the first 2 to 5 months period. During the 2 succeeding seasons of composting in full scale the chlorophenol degradation was only 55% and 10%. The best indicator of the actual chlorophenol degradation was the number of bacteria growing on 2mM pentachlorophenol plates as well as the detection of specific chlorophenol-degrading strains in compost samples by real-time quantitative PCR. Parallel bench scale composts showed that around 60% of pentachlorophenol was mineralised. There were no harmful side reactions such as biomethylation or polymerization and the toxicity to luminescent bacteria decreased with the chlorophenol degradation. We observed, however, that surprisingly high amounts of polychlorinated dibenzo-*p*-dioxins and dibenzofurans were present in the contaminated soil as impurities in the chlorophenolic waste and these remained constant throughout the composting time. The use of straw compost as inoculum did not enhance the chlorophenol degradation in comparison to addition of bark chips to the contaminated soil.

1345 Field application of bioremediation in the UK: pragmatic approaches for achieving clean up

RICHARD J.F. BEWLEY
URS Dames & Moore Blackfriars House, St. Mary's Parsonage, Manchester M3 2JA

A risk-based approach to remedial assessment with the development of pragmatic clean up targets may favour the selection of bioremedial approaches to contamination. Using *in situ* landfarming, with a slow-release nutrient, leachable petroleum hydrocarbons (TPH) at a former oil terminal site

adjacent to a river were reduced to below 1mg/l from previous concentrations of up to 39 mg/l. Similar leachability targets were achieved by an *ex situ* approach at a former lubricating oil terminal. The latter also included an *in situ* remedial scheme involving nutrient injection and oxygenation by hydrogen peroxide to reduce TPH, typically in the hundreds of mg/l, to below 25 mg/l. This was followed by a passive approach to monitor further attenuation, with concentrations after 2 years fluctuating between 1 and 0.1 mg/l. *In situ* remediation of groundwater at a former gasworks site was also undertaken with nitrate as electron acceptor followed by 2.5 years of ongoing monitoring. Mean concentrations of phenols, BTEX and PAHs were respectively reduced to 92, 11 and 0.7 mg/l from starting levels of 1100, 58 and 11 mg/l. Passive approaches may therefore provide cost effective means of managing contamination issues, either employed sequentially with active treatment methods, or as monitored natural attenuation alone.

1430 Bioremediation of DDT-contaminated soil: enhancement by seaweed addition

IAN SINGLETON, DUANGPORN KANTACHOTE, RAVI NAIDU, BRIAN WILLIAMS, NICHOLAS McCLURE and MALLAVARAPU MEGHARAJ
Dept of Soil and Water, Waite Campus, University of Adelaide, Glen Osmond SA 5064, Australia

Prior and continued use of DDT for insect control has led to serious environmental contamination from both DDT and its' primary transformation products DDD and DDE. The recalcitrance of these compounds means that contaminated areas need to be remediated and bioremediation is a potential option. However the limited bioavailability of DDT in soil is a major constraint to bioremediation and the production of DDE during remediation must be avoided due to the known persistence and environmental health concerns associated with this compound. Consequently we investigated the potential of seaweed to increase DDT biotransformation in soil as the material is cheap and acts as a source of Na and organic carbon which are known to affect soil dispersion and therefore potentially increase DDT bioavailability. Long term DDT-contaminated soil was incubated with varying amounts of seaweed under flooded conditions and DDT transformation followed by solvent extraction and gas chromatography. Results indicated that lower levels of seaweed addition significantly increased (20% increase over control) DDT transformation and a level of 0.5 % seaweed (w/w) reduced DDT levels below South Australian EPA guidelines for clean soil within 6 weeks. DDD was the major transformation product found and the presence of DDD transformation products (e.g. DBP) confirmed further metabolism. In conclusion seaweed appears to enhance DDT transformation in DDT contaminated soil and could be used as a potentially cheap material to improve bioremediation. Further work on different soil types and pollutants is necessary to determine the general applicability of seaweed for enhancing bioremediation.

1445 Combining bioremediation with electrokinetics for the treatment of contaminated soil

S.A. JACKMAN[1], G. MAINI[2], A.K. SHARMAN[2], G. SUNDERLAND[3] and C. KNOWLES[1]
[1]Oxford Centre for Environmental Biotechnology, Dept of Engineering Science, University of Oxford, Parks Road, Oxford OX1 3PJ, and Natural Environment Research Council Centre for Ecology & Hydrology, Mansfield Road, Oxford OX1 3SR, [2]Viridian-Ehc Ltd, 114-116 John Wilson Business Park, Thanet Way, Whitstable, Kent CT5 3QT, [3]Ea Technology Ltd, Capenhurst, Chester CH1 6ES

Electrokinetics, the application of direct electric current, has been used to remediate metal and organic contaminated soil for the last two decades. The electric current causes (1) electromigration of cationic metal ions towards the cathode, and (2) an electroosmotic flow of water towards the cathode, transporting dissolved uncharged organics. Initial studies on historically contaminated soil from a former gasworks site demonstrated that PAHs and BTEX compounds were removed efficiently in the presence of surfactants. In contrast, metal ions were not removed from the soil. Focussing upon the remediation of organic contaminants, a model compound, 2,4-dichlorophenoxyacetic acid (2,4-D), was studied. 2,4-D is negatively charged at neutral pH values and is completely mineralised by *Burkholderia* spp. *RASC* c2. When the contaminant was added to silt soil in an electrokinetic reactor, it migrated through the soil towards the anode. When radiolabelled 2,4-D was incorporated adjacent to the cathode and degradative bacteria were introduced to a region near the anode, application of a DC current led to the movement of the pollutant into the inoculated zone. Movement was correlated with biodegradation *in situ* and recovery of $^{14}CO_2$. The potential use of these combined techniques to understand the bioavailability of organic contaminants will be discussed.

1500 Bioremediation: Does it have to be an Empirical Science?

RICHARD SWANNELL[1], IAN HEAD[2], MICHAEL MILNER[2], WILFRED RÖLING[2], KENNETH LEE[3] and SARAH MacNAUGHTON[1]
[1]AEA Technology Environment, Culham, Abingdon, OX14 3DB; [2]Fossil Fuels & Environmental Geochemistry and Centre for Molecular Ecology, Drummond Building, University of Newcastle, Newcastle upon Tyne NE1 7RU; [3]DFO, Mont-Joli, Canada

Bioremediation is an established technique for the treatment of organically contaminated land both in Europe and North America. During a recent field trial an experimental oil spill was established on a shoreline at Stert Flats in the South West of England. Compared to an oiled control, bioremediation of the sediment resulted in significant ($P<0.05$) stimulation of the microbial community and a concomitant degradation of the crude oil. Data from denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified bacterial 16S rRNA gene fragments obtained from the sediment showed that the nutrient addition stimulated predominantly different populations from those obtained from unfertilised sediment. This raised the prospect that bioremediation strategies could be designed to promote the maximal degradation of contaminant by delivering the optimal nutrient levels in accordance with resource ratio theory. This "bioengineering approach" to bioremediation was investigated in a laboratory microcosm study using a fixed concentration of oil and variable nutrient inputs. Results from this study indicated that the microbial populations present in the microcosm treated with oil and 10% N and 1% P were distinct from all those treated with lower levels of nutrients.

POSTERS

EM 01The induction of 4-hydroxyphenylacetic acid-1-hydroxylase and the homogentisate pathway in *Burkholderia cepacia* ATCC 29351 by 4-hydroxyphenylacetic acid

R.Y. HAMZAH and B.S. AL-BAHARNA
Biotechnology Program, Arabian Gulf University

Phenylacetic acid and its hydroxy derivatives are frequently encountered as metabolic intermediates in the degradation of various aromatic compounds, including tropic acid, atropine, aromatic amino acids, aromatic biogenic amines, pesticides, lignin as well as several short and long-chain phenyl alkanes. In view of the wide spread of phenylacetic acid derivatives in nature, the study of the pathways employed by microorganisms in the degradation of such compounds is of great environmental interest. The present report describes the degradation of 4-hydroxyphenylacetic acid by *Burkholderia cepacia ATCC 29351*, which until recently was classified as *Pseudomonas cepacia*. This bacterium has received special interest in literature due to its extraordinary catabolic potential and ability to survive in unfavourable environments. The results obtained indicate the ability of *B. cepacia* to utilize 4-hydroxyphenylacetic acid as a sole carbon source, whereby it is degraded via the homogentisate

pathway into intermediates of the TCA cycle. The initial reaction in 4-hydroxyphenylacetic acid degradation was a NADH-dependent hydroxylation of the benzene ring, accompanied by migration of the side chain to give 2,5-dihydroxyphenylacetic acid (homogentisate). In presence of 2,2-dipyridyl (homogentisate 1,2-dioxygenase inhibitor), a stoichiometric conversion of 4- hydroxyphenylacetic acid into homogentisate was detected spectrophotometrically. The subsequent cleavage of the benzene ring of homogentisate was catalyzed by a Fe^{2+}-dependent 1,2-dioxygenase and the ring fission product was identified as maleylacetoacetate, according to its spectral characteristics. Further degradation of maleylacetoacetate was catalyzed by a glutathione-dependent isomerase yielding fumarylacetoacetate, which was in turn hydrolyzed to give fumarate and acetoacetate. Based on the result obtained, a degradation pathway for 4-hydroxyphenylacetic acid by *B. cepacia* is proposed and compared to those reported in other bacteria. The significance of this pathway in bioremediation and in investigating novel chemical reactions is also highlighted.

EM 02A novel strategy for biological phosphate removal and polyphosphate production from wastewaters

JOHN W. McGRATH, ALAN MULLAN and JOHN P.QUINN
QUESTOR Centre and School of Biology and Biochemistry, Queen's University, 97 Lisburn Rd., Belfast BT9 7BL, N. Ireland

Phosphate (P) is one of the major nutrients contributing to the eutrophication of lakes and other water bodies; its removal from wastewaters following conventional activated sludge treatment is achieved largely by chemical precipitation which is expensive and may increase sludge volumes by up to 40%. An alternative, biotechnological, approach is that of 'Enhanced Biological Phosphate Removal' (EBPR) which exploits the ability of some microorganisms to accumulate P (as polyP) in excess of their normal metabolic requirements. EBPR systems are economically-attractive, but require anaerobic pre-treatment zones and display inconsistencies in performance that necessitate periodic organic matter supplementation and/or chemical 'polishing' to meet compliance limits. Whilst studying the regulation of microbial polyP metabolism we have recently discovered that it is possible to increase the level of P removal by the microflora of conventional activated sludge plants - *under fully*-aerobic conditions - by up to 142% if the operational pH is adjusted to a value within the range 5.5 to 6.5, as opposed to the values between approximately 7.2 and 7.7 that are typical of current practice. Some 30% of individual sludge microbial isolates - bacterial, yeast and fungal – demonstrated such acid-stimulated 'luxury' P uptake: in a Candida humicola isolate a 4.5-fold enhancement of P uptake was accompanied by a 10.5-fold increase in cellular polyP content and 6-fold higher levels of polyP kinase activity. This previously-unrecognised phenomenon is likely to be of considerable importance for P-cycling in the biosphere, but may also have application in new technologies for nutrient removal from wastewaters; it is currently being trialled by us in a 2000-litre pilot plant.

EM 03Economics of composting for organic waste to avoid soil pollution: case study of Maharashtra State, India

LALITKUMAR P. CHAUDHARI
Vice President, Institute for Sustainable Development & Research, N-1-8, Narayan Pujari Nagar , Worli, Bombay, 400018, India

The Maharashtra State in India have 325 Towns and 33 cities. There are 232 municipal councils including A, B, and C class, and 12 municipal corporations. These local authorities are looking after the civic administration at town or city level. These towns are now facing the acute solid waste problem causing the environmental pollution. The combined effect of this pollution is degradation in the soil, water and air quality, which ultimately affect the civic health in these areas. The farmers from the periurban areas are bringing their agricultural products including vegetables in the city markets. This creates large amount of the solid waste especially organic waste which contain more percentage of moisture content. The studies in this paper points to a number of considerations that should be taken in to account when planning the environmental strategies and selecting the policy instruments for solid waste disposal in towns using composting process. The economic instruments for reduction of solid waste can not be successfully implemented with out pre-existing appropriate standards and effective monitoring and enforcements capacities. Although economic incentives have been viewed by some as alternative to the traditional command and control approach, they can not be considered as short cuts to the solid waste management. The source reduction, source separation and producer responsibility, these three factors are critical in developing and designing the model for economic and optimum waste management model using composting system in the towns of the Maharashtra. The municipal councils and local authorities are spending large amount of their budget on solid waste management system, which is major constraint in expanding other civic services to citizens. This paper will deals with present scenario in compost using the organic waste and its application in agriculture and forestry, past efforts to ensure the economic development of compost process from agriculture waste, kitchen waste, market waste and organic waste.It also discusses some of the recent successful examples in regards to public - private and peoples partnership in infrastructure provision for biotreatment and marketing for the same. The paper emphasizes for extensive capacity building for compost process and its application for sustainability at local level for economic use of organic waste

EM 04Promotion of plant growth and nutrient uptake of cotton and wheat by associative bacteria

DILFUSA EGAMBERDIYEVA[1] and GISELA HÖFLICH[2]
[1]Institute of Microbiology, Uzbek Academy of Sciences, Abdulla Kadiry str.7 B, Tashkent 700128, Uzbekistan, [2]Institute for Primary Production and Microbiological Ecology, Centre for Agricultural Landscape and Land Use Research, Eberswalder St. 84, D-15374, Muencheberg, Germany

In pot experiments, the effect of associative bacteria isolated from the root zones of different agricultural crops grown in the calcisol soil of the semi-arid climate of Usbekistan was investigated with respect to the growth and nutrient uptake of cotton and wheat. After inoculation with the bacterial strains Pseudomonas alcaligenes PsA15, Bacillus amiloliguefaciens BcA12 and Mycobacterium phlei MbP18, the root and shoot growth of cotton and wheat increased significantly. Bacterial inoculation also resulted in significantly higher values of nitrogen and phosphorus contents of plant parts. Effective bacteria produced the phytohormone auxin, B-complex vitamins, different proteins and reacted antagonistically to the soil-borne plant pathogens, Fusarium oxisporum and Verticillum loteritum. The bacterial strains are osmotolerant. The bacterial strain Bacillus amiloliguefaciens BcA12 survived after root and shoot inoculation in the rhizosphere and in the soil of the root zone of wheat.

EM 05Effect of rhizosphere on degradation of PCP in batch culture and soil experiments

R.I. DAMS, G.I. PATON and K. KILLHAM
University of Aberdeen, Dept of Plant and Soil, Cruickshank Building, St.Machar Drive, Aberdeen AB24 3UU

In this present work the main aim is the monitoring of the breakdown of the target pesticide in the soil environment and study the influence and effect of rhizosphere on breakdown. The target pesticide is pentachlorophenol (PCP) and the micro-organism choose was *Sphingomonas chlorophenolica* ATCC 39723. PCP is one of the most widely distributed and versatile biocides used as fungicide, insecticide and termiticide and it is lethal to a wide variety of organisms,

both plant and animal, as inhibitor of oxidative phosphorylation (Crosby, 1981; Saber & Crawford, 1985). In batch culture it was used a Mineral Salts Medium (MSM) added of PCP from a stock solution and degradation experiments were carry out in MSM medium and MSM added of rhizosphere extracts (MSM+RHI) at 200 r.p.m and 25°C. Removal of PCP was monitored at 320 nm and concentration of PCP analysed through HPLC. Enumeration of *S. chlorophenolica* ATCC 39723 were made in MSM plates. Soil experiments were carry out with Boyndie soil spiked with PCP (100mg/kg), inoculated with *S. chlorophenolica* ATCC 39723, with growing plants (wheat seeds) or not. Controls were without inoculum. Each treatment had 5 replicates, and the experiment run for 4 weeks. Alicotas (weekly) were taken for PCP quantification (HPLC) and enumeration of *S. chlorophenolica* ATCC 39723. In batch cultures *S. chlorophenolica* ATCC 39723 showed be able to degrade PCP in both media, MSM and MSM+RHI. Rhizosphere showed accelerates the process of biodegradation and the results indicate that rhizosphere has a positive effect on degradation of PCP. In soil experiments, PCP was completely degraded in two weeks time in soil inoculated with *S. chlorophenolica* ATCC 39723 and plant. There was no degradation only with plants without inoculum. Controls remained the same.

EM 06Redox controlled gene expression for use in the bioremediation of phenolic compounds

K. DODGE, E. SCOTT and C. EDWARDS
School of Biological Sciences, Life Sciences Building, University of Liverpool, Liverpool L69 7ZB

FNR (for fumarate and nitrate reduction) and ANR (for anaerobic regulation of arginine catabolism and nitrate reduction) are homologous transcriptional regulators of anaerobic respiration in *Escherichia coli* and *Pseudomonas aeruginosa* respectively. A strategy to construct a redox controlled gene cassette encoding for salicylate hydroxylase (SalA) is being tested. This involves amplification of *SalA* without its promoter using the polymerase chain reaction and insertion downstream of the modified ANR promoter. Degradation of salicylic acid will be monitored under redox conditions that promote ANR-driven gene expression. The efficiency of degradation by the recombinant *Pseudomonas putida* will be compared with natural isolates capable of utilising salicylic acid as a sole carbon source. Natural isolates have also been screened for the distribution of the *salA* gene.

EM 07Analysis of methangen ecology in a hypereutrophic lake using molecular techniques

J. EARL[1], D.A. RITCHIE[1], G.H. HALL[2], R. PICKUP[2], J. SAUNDERS[1] and C. EDWARDS[1]
[1]University of Liverpool, School of Biological Sciences, Life Sciences Building, Liverpool L69 7ZB, [2]Institute of Freshwater Ecology, Windermere, Cumbria

Methane is over 20 times more effective as a greenhouse gas than carbon dioxide, and has been estimated to persist in the atmosphere for 9-12 years, thus making it an ecologically significant gas. Due to their impact on the environment, the study of methanogens from a variety of niches has become common. Their need for strict anaerobic growth conditions makes methanogens difficult to culture. Therefore, the use of molecular techniques allows us to analyse the total population without culture bias. Sediment and water samples were taken on a monthly basis over one year, and subjected to analysis by molecular ecology methods to analyse the methanogen population. In addition, measurements of methane concentration were determined. Methanogen specific DNA was detected in sediment and water samples throughout the sampling year. Analysis of 16S rDNA using TTGE demonstrated a seasonal change in the archaeal population. Surprisingly a more diverse population was present during the winter months. RFLP analysis was also applied to the same samples for analysis of *mcr* gene sequences and was shown to be a useful marker for methanogen diversity.

EM 08Role of flavonoids in rhizoengineering

K. NARASIMHAN, V.S. BHINU and SANJAY SWARUP
Molecular Plant-microbe Interactions Laboratory, Dept of Biological Sciences, National University of Singapore, Singapore-117 543

Rhizosphere supports majority of microbes in the soil yet its potential for manipulating microbial communities has not been highly exploited. The ability to catabolize exotic nutrients exuded by the roots can be used in the successful engineering of host colonization by a bacterium when in competition with others. To test this strategy we designed an experimental model using a *Pseudomonas putida* PGPR strain that utilizes flavonoids, and several flavonoid pathway mutants and a transgenic of *Arabidopsis*, which exude various amounts of flavonoids. Flavonoid accumulation in the root exudates of these *Arabidopsis* lines was studied using RP-HPLC and mass spectrometry. Root colonization of some of these lines by GFP-marked flavonoid-utilizing *Pseudomonas* strain and its non-utilizing mutant were studied. Bacterial counts and confocal microscopy were used to monitor the colonization process. Results indicated that flavonoid-utilizing strain was significantly better than its non-utilizing mutant in the ability to colonize the roots of flavonoid-producing *Arabidopsis* plants. The colonizing abilities of the two strains were comparable for the flavonoid non-producing mutants of *Arabidopsis*. Significantly higher ratios of utilizers to non-utilizers were observed on flavonoid overproducing plants. These findings suggest that engineering flavonoid utilitisation trait into soil microbes can be used to improve their colonization ability.

EM 09Flavonoid catabolism in *Pseudomonas putida*

V.S. BHINU, K. NARASIMHAN and SANJAY SWARUP
Molecular Plant-microbe Interactions Laboratory, Dept of Biological Sciences, National University of Singapore, Singapore-117 543

Enhanced rhizo-colonisation by microbes capable of efficient nutritional resource partitioning can be exploited to improve bioremediation. In order to study root exudate flavonoids as a model nutritional source we have identified and partially characterized a flavonoid-utilizing *Pseudomonas putida* strain. Nine independent flavonoid-auxotrophic mini-Tn5 mutants were created. Studies on the growth characteristics of wild type and the mutants in quercetin-supplemented media revealed the inability of mutants to catabolise flavonoids. In order to understand the degradation pathway, the intermediate compounds differently accumulating in the wild type and mutant strains were studied using a combination of RP-HPLC, Ion spray-Mass Spectrometry and NMR. Culture supernatant, soluble cell fractions and membrane fractions of the strains were used in the analysis. A comparison of accumulated intermediates between the wild type and mutant culture strains allowed us to understand some of the degradation events. A pathway for quercetin degradation involving an early-acting quercetin dehydroxylase and protocatechuate formation is proposed. Cloning of the genes involved in the flavonoid catabolism is underway.

EM 10Influence of sediment-associated natural organic matter on SDS biodegradation

SAMANTHA MARSHALL and GRAHAM WHITE
School of Biosciences, Cardiff University, Biomedical Building, Museum Avenue, PO Box 911, Cardiff CF10 3US

The fate of organic pollutants arriving in river environments is dependent not only on their potential for biodegradation but also on the way in which they interact with sediments. The presence of natural organic matter (NOM) on sediments can greatly enhance the adsorption of hydrophobic pollutant molecules to the surface, which in turn can influence their bioavailability. The influence of sediment-associated NOM on the adsorption and biodegradation of sodium dodecyl sulphate (SDS) by *Pseudomonas* C12B, was investigated. In the presence of native sediment, rich in NOM, SDS was found to adsorb strongly and its biodegradation by *Pseudomonas*

C12B was enhanced in comparison to that observed in the absence of sediment. This enhancement could not solely be attributed to the presence of indigenous bacteria in the sediment. An increase in the ratio of sediment-attached to free-living bacteria was also observed at the onset of biodegradation. In contrast, SDS did not adsorb significantly to organic free sediment and the rate of its biodegradation was comparable to that in the absence of sediment. The increase in sediment-attached to free-living bacteria at the onset of biodegradation was also diminished. Hence, sediment-associated NOM may enhance SDS biodegradation by localising both the surfactant and bacteria at the sediment surface, thereby increasing the rate of catalysis.

EM 11A novel enzyme for biodegradation of the branched primary alkylsulfate surfactant, 2-butyloctyl sulfate

ANDREW J. ELLIS[1], STEPHEN G. HALES[2], NAHEED G. A. UR-REHMAN[2] and GRAHAM F. WHITE[1]
[1]School of Biosciences, Cardiff University, PO Box 911, Cardiff CF10 3US, [2]SEAC Environment Centre, Unilever Research Port Sunlight, Quarry Road East, Bebington, Wirral, Merseyside L63 3JW

The anionic surfactant group embraces a range of compounds distinguished on the basis of chemical structure, and includes primary alkylsulfates such as sodium dodecyl sulfate (SDS). These compounds are based on alcohols produced through the oxo process which yield a mixture of linear (LPAS) and branched (BPAS) primary alkyl sulfates. Branched primary alkylsulfates such as 2-butyloctyl sulfate are also produced via sulfation of the products of the Guerbet reaction. Although BPAS are firmly established as being readily biodegradable, nothing is known about the biochemical basis of biodegradation of this group of anionic surfactants. Standard enrichment techniques were used to isolate bacteria that utilised 2-butyloctyl sulfate as a sole source of carbon. *Pseudomonas* AE-D has been shown to metabolise 2-butyloctyl sulfate and produce a single alkylsulfatase enzyme, DP1, as shown by gel zymography. This alkylsulfatase has been purified to homogeneity and is active towards a range of BPAS, although shows very limited activity towards LPAS and linear secondary alkyl sulfates (LSAS). Other characteristics, including catalytic and molecular properties, of the DP1 alkylsulfatase have also been investigated.

EM 12Complete denitration of glycerol trinitrate by bacteria isolated from a wash - water soakaway

SAMANTHA MARSHALL and GRAHAM WHITE
School of Biosciences, Cardiff University, Biomedical Building, Museum Avenue, PO Box 911, Cardiff CF10 3US

Glycerol trinitrate (GTN) is used as both an explosive for military and civilian applications and also as a treatment for heart disease. During its production, waste wash-waters saturated with GTN have commonly been transferred to lagoons or soakaways, resulting in actual or potential contamination of soils/sediments. Several bacterial strains capable of GTN biodegradation were isolated from a wash-water soakaway. When grown in the presence of glycerol as a C source and GTN as the sole N source, most of these strains were capable of removing either one or two nitro-groups from GTN, thereby producing dinitrates and mononitrates. However, one isolate, identified as a Rhodococcus species, was shown not only to biodegrade GTN rapidly, but also to remove all di- and mononitrates formed, and thus achieve complete denitration of GTN. Complete denitration of GTN by the *Rhodococcus* sp. was consistent with its ability to utilise GTN as a combined sole source of both C and N. Of the other isolates, a *Pseudomonas putida* strain was also shown to utilise GTN as sole sources of N and C, despite accumulating glycerol mononitrates when grown in the presence of glycerol, suggesting that the removal of the final nitro-group may be nutrient-regulated. This observation may have important implications in the field of bioremediation, where alternative nutrients are often added to boost bacterial growth but which may have regulatory influences on the biodegradation of pollutants.

EM 13Conditional sensitive whole cell biosensors containing a novel reporter for the detection of pollutants in soil and water

J. MEADOWS, J. FIRTH, E. SCOTT and C. EDWARDS
School of Biological Science, The University of Liverpool, Life Science Building, Crown Street, Liverpool L69 7ZB

Bacteria can exhibit altered pathways of gene expression when exposed to various environmental conditions and these pathways can be exploited to provide pollutant indicators. The potential of pollutant sensitive promoters to drive the expression of a novel reporter gene, the *din* gene that encodes a DNA invertase responsible for inversion of a specific segment of DNA will be described. On exposure to an appropriate pollutant, a DNA inversion should be induced that can be used as a permanent record for a pollutant's presence. DNA inversion will be detected by a combination of restriction enzyme analysis and PCR. A number of general cloning strategies have been employed to construct a series of pollutant biosensors including ones for toluene, benzoate and mercury which contain both *din* and a green fluorescent protein as comparable reporters. PCR based cloning methods are in progress to construct the plasmid based reporter systems, stable chromosomal insertion of the biosensor genes into *Pseudomonas* as well as testing of recombinant bacteria in laboratory scale microcosms to mimic the situation in natural environments.

EM 14Studies towards the bioremediation of nitrile and metal cyanides

N.J. GARTON[1], S.P. CUMMINGS[1], G.W. BLACK[2] and J. COLBY[1]
[1]School of Sciences, The University of Sunderland, Sunderland SR2 3SD, [2]School of Applied and Molecular Sciences, University of Northumbria at Newcastle, Newcastle upon Tyne NE1 8ST

The redevelopment of derelict industrial land is a target of many urban regeneration schemes. Many brown-field sites are contaminated with nitriles (organic cyanides) and metal cyanides which are extremely toxic and can persist in the environment. Nitriles are found as a result of release from industry and in soil as a result of herbicide application. Metal cyanide wastes contaminate disused coke and gas work sites. Currently, such wastes require expensive physical and chemical treatment prior to land re-development. Microbial bioremediation of industrial wastes is used increasingly as a cost-effective method for the removal of certain amenable pollutants. The aim of this project is to investigate the soil bioremediation of metal cyanides and nitriles using whole cells and cell free extracts. Metal cyanide and nitrile degradation by *in-situ* soil microorganisms and specific enrichment isolates have been monitored in culture. Bacteria which utilise a range of metal cyanides (including an iron cyanide complex which is a major contaminant of gas and coke work sites) have been isolated. The nitrile hydratase of *Rhodococcus* AJ270 has been expressed in *E. coli* and any improvement of activity against particularly recalcitrant nitriles will be investigated after *in vitro* mutagenesis techniques.

EM 15Degradation of 2,4-dichlorophenol by a Distal *Meta*-cleavage pathway

KAREN M. O'REILLY and EVELYN M. DOYLE
Dept of Industrial Microbiology, University College Dublin, Belfield, Dublin 4

Chloroaromatic compounds are classed as a major source of priority pollutants due to their recalcitrant nature and detrimental effect on the environment. It is generally accepted that mineralisation of haloaromatics, unlike methylaromatics such as toluene, does not proceed via a *meta*-cleavage pathway due to the toxicity of some of the intermediates. S*phingomonas sp.* IMD 432 utilises a wide range of chlorinated aromatics and readily degrades 2,4-

Dichlorophenol (2,4-DCP) in liquid culture as sole carbon source. Studies on the degradation pathway have revealed that the organism metabolises 2,4-DCP via 3,5-dichlorocatechol and produces both intra- and extradiol dioxygenases when grown on 2,4-DCP. The extradiol dioxygenase is active on a range of chlorocatechols including 3-chlorocatechol (3-CC). Extradiol cleavage of 3-CC generally results in enzyme inactivation due to the formation of an acylchloride. Initial studies suggest that the productive conversion of 3-CC by this *Sphingomonas* sp enzyme is due to the fact that the enzyme catalyses distal rather than proximal cleavage of the chlorocatechol. The enzyme was purified to homogeneity and relevant physicochemical properties examined. The fact that the organism degrades mixtures of chlorobenzene and toluene suggests that it has potential in the degradation of wastes containing both chlorinated and methylated aromatics.

EM 16Biodegradation and biotransformation of fluorinated aromatic compounds

SARAH J. BROOKS, SARAH E. DUNNE, KEVIN E. O'CONNOR and EVELYN M. DOYLE
Dept of Industrial Microbiology, University College Dublin, Belfield, Dublin

Microbial degradation of halogenated aromatic compounds has been concerned to-date with the elucidation of biochemical pathways with a view to developing bioremediation processes. However, many of the microbial systems developed for bioremediation also have potential applications in the production of high value-added products for the fine chemical and pharmaceutical industries, e.g. naphthalene dioxygenase / styrene monooxygenase in indigo production. Although fluorinated hydrocarbons are not major environmental contaminants they are used in the medical industry and as biocides. In addition, metabolites from microbial degradation of fluorinated compounds, such as hydroxylated fluoro-substituted aromatic compounds have applications in the pharmaceutical and plastic industries. Very little information exists on the biochemical routes for fluorinated aromatic degradation compared to the wealth of information available on chlorinated compounds. This work undertakes, for the first time, a systematic investigation of the pathways of fluorinated aromatic degradation. A number of bacteria have been isolated from soil which are capable of degrading fluorobenzene and fluorobenzoic acid and the rate at which selected strains transform fluorobenzene has been determined. In addition, a previously characterised *Pseudomonas* sp. with phenol oxidase activity was shown to transform *p*-fluorophenol in a cell free system. This information will facilitate the development of novel biocatalysts for the production of hydroxylated fluorobenzenes.

EM 17Diversity of methane oxidizing bacteria in a hyper eutrophic lake

R.J. HOLT, G.H. HALL, R.W. PICKUP, D.A. RITCHIE, J.R. SAUNDERS and C. EDWARDS
School of Biological Sciences, Life Sciences Building, Crown Street, University of Liverpool, Liverpool L69 7ZB

The diversity of methane oxidizing bacteria has been studied in Priest Pot, a hyper eutrophic lake situated in the Lake District, UK. Priest Pot is a small (1 ha), shallow (3.5m) lake that exhibits an annual stratification pattern due to the nature of its sheltered location. In summer the lake contains 3 distinct layers; a lower anaerobic layer, a smaller middle layer that contains a temperature and oxygen gradient and an upper aerobic layer. In winter these layers dissipate giving rise to a uniform temperature and oxygen gradient. Other studies have utilized several different genes to ascertain the diversity of methane oxidising bacteria in a variety of different environments including; 16s rRNA genes and both particulate and soluble methane monooxygenase genes (PMMO and SMMO). In this study a combined approach was used and primers for both 16s and PMMO genes were used. 4 sets of 16s primers were used which had been designed to detect the sequences of *Methylobacter*, *Methylococcus*, *Methylomonas* and *Methylosinus* species of methanotrophs. The primers used to detect the PMMO gene had been designed so as not to detect the ammonia monooxygenase gene (*amoA*), unlike several primers which have been previously described. Samples were taken from Priest Pot for a total of 13 months at monthly intervals to ascertain if an annual pattern of diversity could be observed. Samples were taken from surface water and at 1, 2 and 3 metre depths and sediment samples were taken at 0, 1, 2, 3, 5, 10 and 14 cm. In addition to this, temperature and oxygen profiles were obtained to ascertain if a thermocline/oxycline was present. Methane and methane oxidation levels were also measured to determine if these had any effect on diversity. DNA was extracted from all samples and tested using PCR with both the 16s and PMMO primers. This showed that methane oxidizing bacteria were not present in winter months (Oct-March) but were present in the summer months. PCR using primers designed to detect the *mxaF* gene, which is present in methylotrophs was however detected in samples from both winter and summer months.

EM 18Fungal transformation of metal-bearing minerals

JONATHAN P. MOSES and GEOFFREY M. GADD
Division of Environmental and Applied Biology, School of Life Sciences, University of Dundee, Dundee DD1 4HN

The ability of several fungal isolates to tolerate and solubilize the metal-bearing phosphate minerals apatite (Ca5(PO4)3OH) and pyromorphite (Pb5(PO4)3) was investigated. Three of the isolates tested (*Aspergillus niger*, *Serpula himantioides* and an unidentified basidiomycete) were able to solubilize apatite while five (*A. niger*, *S. himantioides*, the unidentified basidiomycete, a *Penicillium* sp., and *Poria placenta*) were able to solubilize pyromorphite. Concomitant with mineral solubilization was the production of calcium oxalate crystals during the solubilization of apatite and lead oxalate crystals following the solubilization of pyromorphite. Concentrations of calcium in the fungal biomass increased with increasing concentrations of apatite in the medium while biomass lead concentrations increased with increasing concentrations of pyromorphite in the medium. These results are discussed in the light of their relevance to land reclamation, nutritional heterogeneity and metal and phosphate biogeochemistry.

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EM 19Interactions between toxic metal/organic mixtures assessed using the bioluminescent response of a *Lux*-modified bacterium

STUART C. SNEDDON and GEOFFREY M. GADD
Division of Environmental and Applied Biology, School of Life Sciences, University of Dundee, Dundee DD1 4HN

Contamination of the environment with large numbers and quantities of pollutants has strengthened the need to assess the toxicity of particular pollutants and their potential ecological impact. The current extent of contamination means that many polluted sites contain mixtures of pollutants, the combined toxicity of which is commonly assumed to exhibit additivity. However, pollutants in a mixture may interact, resulting in toxicity significantly greater than (synergism), or less than (antagonism) the predicted additive toxicity. In this study, the acute toxicities of Cd^{2+} and phenol were assessed individually and in combination, using the bioluminescent response of a *lux*-marked bacterium as an indication of toxicity. Observed bioluminescent responses to toxicant combinations were compared with predicted responses determined by exposure of the test organism to the chemicals individually. The bioluminescent response of *Escherichia coli* HB101 pUCD607 upon exposure to Cd^{2+}/ phenol combinations demonstrated that most mixtures were more toxic in combination than individually, but many were less toxic than predicted. In addition, some combinations exhibited less toxicity than their respective individual controls. These results indicate a concentration-dependent antagonistic interaction between Cd^{2+} and phenol towards the bioluminescent response of *E. coli* HB101 pUCD607.

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EM 20A P-Type ATPase involved in copper resistance in a plant growth promoting *Pseudomonas* strain

V. ADAIKKALAM and SANJAY SWARUP
Molecular Plant-microbe Interactions Laboratory, Dept of Biological Sciences, National University of Singapore, Singapore-117 543

Metals play a vital role in being a part of several functional processes in living systems. Copper is one of them as being a cofactor for key enzymes such as cytochrome c-oxidase, hemocyanin and superoxide dismutase. However, cells need to keep the intracellular concentration of free copper ion low since the free metal ion can facilitate oxidative damage leading to lethality. Therefore, the entry of copper into the cell, intracellular trafficking and maintenance of optimum copper level are crucial to normal cellular metabolism. We were interested to understand the genetics of copper homeostasis, and the mechanism of copper resistance in a plant growth promoting rhizobacterial *Pseudomonas* strain. Mini-Tn5(gfp) transposon mediated mutagenesis was used to tag copper-responsive genes. One mutant responsive to copper was found to be handicapped in copper transport. The transcriptionally fused GFP gene in the mutant was used as a means to study the resident gene expression under copper limiting and excess conditions. The minimal inhibitory concentration of copper for the wild type and the mutant bacteria were 70µM and 300µM respectively in MGY medium. The gene was identified as a P-type ATPase involved in copper transport.

EM 21An Antarctic Bacterium capable of degrading aromatic hydrocarbons

BETHAN STALLWOOD[1], JOHN SHEARS[2] and PETER A. WILLIAMS[1]
[1]School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd, [2]British Antarctic Survey, High Cross, Madingley Rd, Cambridge

A psychrotolerant bacterium, ST41 has been isolated from oil-contaminated soils on Signy Island, Antarctica. The bacterium degrades a wide range of aromatic compounds at low temperatures. The 16S rDNA sequence shows that ST41 is a Pseudomonad, having 97% homology to the gene from *Pseudomonas syringae*. Genes from ST41 involved in the degradation of aromatic compounds have been cloned and sequenced. The cloned genes show close similarity to those in the *meta* pathway operon from the TOL plasmid pWW0 from the mesophilic *P. putida* mt-2 . Growth of ST41 on benzoate results in a high frequency of segregants which are unable to grow on the TOL plasmid substrates that are degraded via the *meta* pathway. This suggests that ST41 carries a TOL-like plasmid, although there is no direct evidence from either plasmid isolation or interspecific tranfer of the genes to substantiate this. ST41 is able to degrade aromatic compounds at 0°C whereas mesophilic bacteria cannot. Its growth on oil constituents at low temperatures has shown that ST41 has the potential to be used in bioremediation of oil spills on Antarctica and bioaugmentation experiments in microcosms are underway to test its capacity at low temperatures.

EM 22Isolation and characterization of microorganisms degrading quaternary aminoalcohols - hydrolysis products of esterquat surfactants used as laundry softeners

A. KAECH and T. EGLI
Umweltmikrobiologie und Molekulare Oekotoxikologie, EAWAG, Duebendorf, Switzerland

Today, mainly three structurally similar esterquat surfactants are used as laundry softeners in washing detergents. All of the three surfactants appear to hydrolyse rapidly (probably abiotically) when reaching surface water or sewage treatment plants, providing the corresponding fatty acids and three quaternary aminoalcohols (QAA's) as products. These three QAA's are trimethyl-2,3-dihydroxy-propyl-ammonium, dimethyl-diethanol-ammonium and methyl-triethanol-ammonium. The similarity of the QAA's and to the naturally occurring compound choline suggests that all three QAA's should degrade in a similar way and with a similar rate comparable to choline. However, despite this similarity they show different degradation pattern as well as different degradation rates in OECD die-away tests. No organisms have yet been isolated able to degrade QAA's and the catabolic pathway for those compounds have not been elucidated yet. Therefore, we have set out to enrich and isolate microbes able to grow with different QAA's as their only source of carbon, energy and nitrogen. Microorganisms growing with the different QAA's as sole source of carbon, energy and nitrogen were enriched from soil, river water and sewage sludge. Five strains were isolated and several tests (Api 20 NE, Biolog, 16S-rDNA sequencing etc.) were performed to compare the isolates. Four out of five QAA-degraders were able to utilise only one specific QAA. All of the isolated microorganisms were able to grow with the natural compound choline. On the other hand no choline degrader, enriched with choline as sole carbon, energy and nitrogen source, was able to degrade the tested QAA's.

EM 23The *in situ* microbial degradation of soil buried polyurethanes by biofilms

SARAH BARRATT[1], PAULINE HANDLEY[1], GEOFF ROBSON[1], IAN EASTWOOD[2] and MARIANNE NIXON[2]
[1]University of Manchester, 1.800 Stopford Building, Oxford Road, Manchester M13 9PT, [2]Avecia Biocides, Blackley, Manchester

Polyurethanes (PUs) can persist in the natural environment for many years and the prime site for their disposal is in landfill sites. The aim of these experiments was to assess the effect of soil water content on the rate of PU degradation. The soil water content was adjusted to 22.5% water holding capacity (whc) or 60.0% whc and polyester and polyether PU were buried in soil for 44 days. Tensile testing measurements showed a 24% decrease in elasticity of polyester PU buried at 22.5% whc compared to the unburied negative control, whilst all other samples and conditions did not show signs of degradation. Biofilm organisms were isolated from the surface of the PU using soil extract agar (SEA) and polyurethane agar (PUA). Fungal degraders on PUA compared to the total fungi on SEA were 95% higher on the surface of polyester PU buried at 22.5% whc compared to all other conditions tested, indicating the utilisation of polyester PU as a nutrient source at 22.5% whc. The fungal numbers were significantly reduced on polyester PU at 60.0% whc. Bacterial numbers did not vary with changing soil water content or PU type, indicating a non-essential role in the initial degradative event. Fungal isolates (5) capable of degrading PUA as a sole carbon source were identified using molecular methods. Fungi were therefore identified as the main effectors of degradation with optimum degradation occurring at the low whc of 22.5%.

EM 24Cloning and molecular characterisation of a novel indole dioxygenase gene from a *Pseudomonas alcaligines* strain which is essential for fluoranthene degradation

LINDA GORDON and ALAN D.W. DOBSON
Dept of Microbiology, National University of Ireland, Cork

Polycyclic aromatic hydrocarbons (PAH) consist of two or more fused benzene rings and enter the environment as a result of the combustion of fossil fuels and as by-products of petroleum processing, where their presence is a cause for concern due to their known toxigenic, mutagenic and carcinogenic properties (1). We have isolated a *Pseudomonas alcaligenes* strain PA-10 which degrades the high molecular weight PAH fluoranthene; in addition to the lower molecular weight PAH napthalene, fluorene, anthracene and phenanthrene. The fluoranthene degradative pathway employed by strain PA-10 appears similar to that previously

described for *Mycobacterium* PYR-1 (2). Several of the genes encoding dioxygenases which act on low molecular weight PAH have been cloned from a number of *Pseudomonas* species and exhibit a large degree (>90%) homology. However, there is considerably less information available concerning genes involved in the catabolism of higher molecular weight PAH such as fluoranthene. We have cloned an indole dioxygenase from *P. alcaligines* which shows no homology with previously cloned dioxygenase genes from *Pseudomonas*. The gene which shows some similarity (24% identity at the amino acid level) to a napthalene dioxygenase from *Rhodococcus* opacus (3). The product of this gene is essential for fluoranthene degradation since a chromosomal mutant of strain PA-10 where the gene has been disrupted fails to metabolise the PAH. In addition catabolism of other PAH is also affected. Thus it appears that this enzyme encodes a novel dioxygenase, with a broad substrate specificity. The isolation of this, and novel genes from other species-such as *Comamonas testosteroni* GZ39 (4) indicates that a far greater genetic diversity exists among PAH degradative microorganisms than many studies have shown.
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EM 25Transcriptional regulation of styrene degradation in *Pseudomonas putida* strain CA-3

NIALL D. O' LEARY[1], KEVIN E. O'CONNOR[2], WOUTER DUETZ[3] and ALAN D.W. DOBSON[1]
[1]Dept of Microbiology, National University of Ireland, Cork, Ireland, [2]Dept of Industrial Microbiology, National University of Ireland, Dublin, Ireland, [3]Institut fur Biotechnologie, ETH Honggerberg, CH-8093 Zurich, Switzerland

Styrene, a toxic aromatic hydrocarbon and potential carcinogen, is released into the environment in large quantities by the petrochemical and polymer processing industries. Research into styrene biodegradation over the last decade has identified a wide variety of bacterial and fungal environmental isolates capable of utilising this compound as a sole source of carbon. The most significant insights into the specific mechanism(s) of styrene catabolism have however come almost exclusively from the genus *Pseudomonas*. Among the *Pseudomonads* thus far examined in depth there exists a high degree of conservation, both in sequence and structural organisation, of the genetic elements involved in styrene degradation. Unfortunately the paucity of information regarding the regulation of these elements leaves substantial questions unanswered regarding potential styrene bioremediation/biotransformation strategies. To this end we have cloned the genes responsible for styrene degradation in *Pseudomonas putida* CA-3 and monitored their regulation under differing physiological conditions in both batch and continuous culturing systems. The styrene degradative pathway in *CA-3* has previously been shown to be divided into an upper pathway involving the conversion of styrene to phenylacetic acid and a lower pathway for the subsequent degradation of phenylacetic acid. We report here that expression of the regulatory genes *sty* S and *sty* R is essential for transcription of the upper pathway, but that degradation of the lower pathway inducer, phenylacetic acid, does not appear to be under their influence. In batch culture experiments the presence of phenylacetic acid in the growth medium completely represses the upper pathway enzymes, even in the presence of styrene, the upper pathway inducer. This repression is mediated at the transcriptional level by preventing the expression of the sty S and sty R regulatory genes. Conversely, during continuous culturing experiments with a limiting concentration of phenylacetic acid, (5mM), as the sole carbon source extremely high levels of *sty* S and *sty* R transcription and corresponding upper pathway activity are observed. Continuous culturing also highlights the moderating effects of inorganic nutrient limitations, (such as may be encountered in the environment); on transcriptional activity of the upper and lower pathways. Finally we examine the various stages of the diauxic growth pattern observed when *P. putida* CA-3 is grown on styrene or phenylacetic acid together with an additional carbon source such as citrate and report that catabolite repression may involve a different mechanism of regulation than transcriptional repression by an additional carbon source.

EM 26 *In situ* molecular site Assessment of diverse PAH catabolic genes using RT-PCR single-strand conformation polymorphism

JAMES E.M. STACH and RICHARD G. BURNS
Research School of Biosciences, University of Kent at Canterbury, Kent CT2 7NJ

A major goal of microbial ecology is the identification of genes that are responsible for the metabolism of xenobiotics in soil. We have developed a procedure for monitoring, in-situ, real-time, polycyclic aromatic hydrocarbon (PAH) gene expression from RNA and DNA directly extracted from PAH-contaminated soil. Furthermore, we assessed the sequence diversity of recovered amplicons using single-strand conformation polymorphism (SSCP). Primers for PCR were used that covered all known PAH-degrading genes. Comparison of SSCP profiles generated from soil DNA or RNA with pure culture representatives indicated that there were diverse PAH catabolic genes being transcribed at the time of sampling. These results were in contrast to conventional culture based techniques which only resulted in *Pseudomonas*-like PAH-catabolic genes being recovered. Transcribed genes were identified as belonging to bacteria from the *Actinobacteridae* and the β, γ subdivisions of the proteobacteria. This technique allows characterisation of catabolic gene transcripts without the need to clone and sequence amplicons, and is therefore compatible with rapid molecular site assessment of soils contaminated with xenobiotic pollutants.

EM 27Aromatic compound catabolism by a novel acidophilic bacterium

KEVIN B. HALLBERG, D. BARRIE JOHNSON and PETER A. WILLIAMS
School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

During the development of a bioremediation system for the treatment of acidic effluents containing both organic and inorganic pollutants, a novel acidophilic bacterium called WJB-3 was isolated due to its ability to grow on benzoic acid at relatively high concentrations (1-10 mM): by comparison other acidophiles were only able to grow on < 200 ?M. WJB-3 was also found to grow on a wide range of aromatic compounds including some substituted phenols. Phylogenetic analysis based on the 16S rRNA gene showed that WJB-3 is an ?-*Proteobacterium* most closely related to two known acidophiles, both members of the genus *Acidocella*. Biochemical studies into the degradation of phenol, a component of our model industrial effluent, by WJB-3 have been carried out. Phenol-grown bacteria express catechol 2,3-dioxygenase (C23O) activity during the degradation of phenol. Two different genes for a C23O have been cloned from WJB-3, one of which is involved in degradation of phenol as shown by its substrate specificity. The phenol degradation operon from WJB-3 differs in structure from the classical phenol degradation operon, the *dmp* operon of *Pseudomonas putida*. This study represents the first on aromatic catabolism by an acidophilic bacterium that has potential for bioremediation of acidic industrial effluents.

EM 28Novel acidophiles isolated from a constructed wetland receiving acid mine drainage

KEVIN B. HALLBERG and D. BARRIE JOHNSON
School of Biological Sciences, University of Wales, Bangor (UWB), LL57 2UW

A pilot-scale constructed wetland site has been in operation since 1993 to treat acid mine drainage (AMD) from the Wheal Jane tin mine in Cornwall, UK. A research consortium, including geochemists, hydrologists and microbiologists, has been established to study the processes which underlie passive treatment of AMD at the Wheal Jane site. As part of this study, we have developed novel solid media for the enumeration of microbes present in the treatment system. A group of isolates were obtained that catalyse the oxidation of ferrous iron and which grow at pH 3-5. These "moderate acidophiles" outnumbered the more familiar extremely acidophilic iron-oxidising microbes such as *Leptospirillum ferrooxidans* in the source AMD and in the surface water and sediment samples from the aerobic cells. Again using novel solid media, heterotrophic microorganisms previously unobserved in AMD were isolated. Phylogenetic analysis based on 16S rRNA gene sequence showed that the iron-oxidisers are unrelated to other acidophilic iron-oxidisers and revealed that the most dominant heterotrophic microorganisms include a novel *Acidobacterium* species and *Propionibacterium acnes*. Using the same solid media, similar isolates have been found in AMD from other metal mines as well as in less acidic mine water from abandoned coal mines. It is essential that the dominant microbes in acidic waters can be cultivated in order to fully understand the role they play in the remediation of AMD, ultimately leading to rational design and operation of AMD bioremediation systems.

EM 29A molecular biological approach to the characterisation of a low level radioactive waste disposal site

R. LOCKHART[1], P. HUMPHREYS[2], I. BEADLE[2] and A.J. M^{C}CARTHY[1]
[1]School of Biological Sciences, Life Sciences Building, The University of Liverpool, Liverpool L69 7ZB, [2]British Nuclear Fuels plc, Risley, Warrington, Cheshire WA3 6AS

Molecular biological techniques have been applied in order to characterise a number of the key microbial groups responsible for the degradation of waste within a low level radioactive waste (LLW) disposal site. This site, at Drigg in the N.W of England, is owned and operated by BNFL and is the principal site for the disposal of LLW in the UK. It has been receiving waste since 1959, with 900,000 m^3 of waste disposed of to date. Historically waste was tumble-tipped into shallow trenches cut into the local geostrata, with a natural low-permeability layer forming the base. As with other LLW sites around the world, the trenches at Drigg are known to be microbially active, with gas profiles showing methane production in some areas of the trenches. Microbial diversity in landfills is considerable and this project has focused on three of the "key" functional groups: sulphate-reducing bacteria, methanogens and cellulolytic bacteria. A number of samples were taken from specific areas of the site and analysed to determine the presence or absence of these three groups of microorganisms. DNA was extracted from all samples and amplified using specific PCR primers, and oligonucleotide probes were used to confirm the identity of the PCR products obtained. Further analysis by Temporal Thermal Gradient gel Electrophoresis (TTGE) was used to assess and identify the species diversity. The data was confirmed by DNA sequencing to generate a profile of the anaerobic biodegradative community active in this radioactive waste disposal site.

EM 30Influence of macroelement limitation on the competitive success of organophosphate degrading bacteria

WANDER SPRENGER[1], ANNEREINOU DIJKSTRA[2], GABRIEL ZWART[3], JOHN PARSONS[1] and PAUL VAN NOORT[2]
[1]Dept of Environmental and Toxicological Chemistry, University of Amsterdam, The Netherlands, [2]Institute for Water Management and Wastewater Treatment, Lelystad, The Netherlands, [3]Institute for Freshwater Ecology, Nieuwersluis, The Netherlands

The efflux of pesticides into surface waters in areas of intensive agriculture pose a threat to aquatic organisms as well as, possibly the production of drinking water. The rate of elimination of these pollutants may differ up to three orders of magnitude for the same chemical compound between different locations(1). Therefore, it is crucial to elucidate what factors determine the competitive success of aerobic organoheterotrophic microorganisms that are capable of bioconversion of pesticides into less toxic products. 400 ml continuous cultures were inoculated with ditch water, and heterotrophic aerobic microorganisms were cultivated for extended periods of time under carbon-, nitrate- or phosphate limitation. In some of the experiments, Flavobacterium sp. ATCC27551, known to be capable of growth on the insecticide diazinon, was introduced into the cultures. The presence of Flavobacterium was monitored by measuring the (enhanced) organophosphorus-degrading activities and by selective plating methods. In addition, the shifts in species composition in the mixed cultures were studied by Denaturing Gradient Gel Electophoretic (DGGE) analysis of PCR-amplified rRNA gene fragments. The effect of addition of the diazinon degradation product 2-isopropyl-6-methyl-4-pyrimidinol to the chemostat medium (a compound that can be used by strain ATCC27551 as the sole source of carbon, nitrogen and energy) on the retention of the strain in the cultures was also investigated.

1) Vink JPM, VanderZee SEATM, WATER RESEARCH 31: (11) 2858-2868 1997

EM 31Comparative physiology of *Thiobacillus prosperus* and *Acidothiobacillus ferrooxidans* at elevated salinity

PATRICIA E. LINTON and J. GRANT BURGESS
Dept of Biological Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS

Bacterially mediated mineral processing is increasingly being employed in the recovery of metals from low grade ores. However, growth of traditional bioleaching bacteria such as *Acidothiobacillus ferrooxidans* and *Leptospirillium ferrooxidans* is significantly decreased by high levels of chloride salts (above 0.1M NaCl) which can contaminate some mining systems. There is therefore a need to assess whether halotolerant metal-mobilising bacteria can be used in biomining technology. Such bacteria would enable the use of salt contaminated ores and brackish water in the process, in areas where freshwater may be scarce. In this study, growth and iron-oxidation by a marine species *T. prosperus* and a freshwater species *A. ferrooxidans* were compared at increasing salinities and when grown on different iron containing substrates. This was carried out using direct cell counts, measurement of ferrous iron levels spectrophotmetrically using chelation with 1,10-phenanthroline and analysis of copper solubilisation rates in a bench-scale study. *T. prosperus* grew and oxidised ferrous iron optimally at approximately 0.2M NaCl when grown on ferrous sulphate whereas *A. ferrooxidans* grows very slowly at this salinity. However, at elevated salinities *A. ferrooxidans* has a higher growth rate when grown on chalcopyrite and chalcocite rather than on ferrous sulphate. The ability of a marine species to oxidise iron and solubilise copper from ores at elevated salinity highlights the potential use of marine acidophilic bacteria in metal bioleaching processes under these conditions.

EM 32Enhanced biodegradation of phenanthrene in cyclodextrin-amended soils

JOANNA D. STOKES, BRIAN J. REID and KIRK T. SEMPLE
Dept of Environmental Science, Lancaster University, Lancaster LA1 4YQ

It is widely accepted that organic compound availability decreases with increasing compound-soil contact time - a phenomenon termed *ageing*. Previous studies have highlighted cyclodextrins as a class of chemicals that can be used to enhance the degradation of hydrophobic pollutants in liquid culture and also increase the concentration of POPs in the aqueous phase. They have also been employed in remediation (pump and treat) strategies. This study investigated the ageing of [9-^{14}C]phenanthrene (a representative persistent organic pollutant) applied at 25 mg kg^{-1} to soil. In particular the influence of hydroxypropyl-β-cyclodextrin (HPCD) on ageing was assessed. [9-^{14}C]Phenanthrene spiked soil was amended with HPCD at a range of concentrations. The results indicated that an increase in soil-phenanthrene contact time, both in the presence and absence of HPCD, resulted in decreased extractability and bioavailability. Additionally, the data suggest that lower concentrations of HPCD retarded this ageing process. This retardation was observed in terms of lower extents of non-extractable residue formation and elevated extents of mineralisation after ageing. Conversely, soil amended with the highest concentration of HPCD exhibits enhanced loss with ageing time. Consequently, there was an enhancement in the formation of non-extractable residues, while bioavailability was decreased greatly. These results have important implications for the treatment of soils contaminated with organic compounds. Application of cyclodextrin solutions to such sites may improve the potential for bioremediation, by retarding ageing processes and increasing the bioavailable fraction. However, cyclodextrin application concentrations would have to be optimised.

EM 33A comparison of the impact that two cable insulating oils have on the soil microbial community

PHILIP H. LEE[1], STUART CLARKE, ALISTAIR W.J. MORRISS[1], DAXABEN PATEL[2] and KIRK T. SEMPLE[1]
[1]Dept of Environmental Science, Institute of Environmental and Natural Sciences, Lancaster University, Lancaster LA1 4YQ, [2]Cables and Transformers, The National Grid Company plc, Leatherhead, Surrey

Light non-aqueous phase liquids (LNAPLs) are used by the electrical generation and transfer industries in high voltage underground electricity cables. Until relatively recently these oils consisted of a mineral oil fraction and a linear alkylbenzene (LAB) fraction. With the increasing cost and supply difficulties associated with mineral oil, a 'pure' LAB oil was introduced. This consists of a range of LAB compounds (C_8-C_{13}) with none of the mineral oil fraction. With this shift in usage it is important to understand the likely affect of any leakage of the oil will have upon the soil environment. To compare the affects of the two different insulating oils have upon the soil microbial community an experiment was run using ^{14}C-glucose mineralisation as an indication of the impact of the two oil types. It was found that the mineralisation rate in the newly introduced pure LAB insulating oil was significantly lower than in the LAB mineral oil mix. The results are of importance in terms of contaminated site remediation. At present 'remediation' is achieved by removal of the soil to a landfill site. If the oil is seen to have an impact on the microbial community, namely a beneficial impact then this may lead to bioremediation be used as a viable site clean-up technology.

EM 34Linear alkylbenzene –microbe interactions in the soil environment

ALISTAIR W.J. MORRISS[1], STUART CLARKE, PHILIP H. LEE[1], DAXABEN PATEL[2] and KIRK T. SEMPLE[1]
[1]Dept of Environmental Science, Institute of Environmental and Natural Sciences, Lancaster University, Lancaster LA1 4YQ, [2]Cables and Transformers, The National Grid Company plc, Leatherhead, Surrey

Light non-aqueous phase liquids (LNAPLs), are commonly found at sites contaminated with coal tar and petroleum fuels, and have been shown to complicate site characteristics in terms contaminant biodegradation and bioremediation. Linear alkylbenzenes (LABs), a class of LNAPLs, are common soil contaminants. An experiment was conducted to investigate the toxicity of a LAB mixture in the soil environment over time. At set time points a substrate induced respiration (SIR) assay, using ^{14}C glucose, was run to assess the impact of the LAB on the soil microbes. At the same time earthworm and lettuce seedlings assays were run. Over time the LAB appeared to increase in toxicity for the earthworm and seedling assays but for the SIR there was little difference in the observed mineralisation rates. To address this question another set of experiments were run using biotic and abiotic systems. Results showed that the abiotic systems were less toxic to the earthworm and seedling assay than the biotic system. These results suggest that the observed increase in toxicity is caused by the soil microbes and maybe due to the incomplete biodegradation of the LAB resulting in the production and accumulation of a toxic metabolite in the soil environment.

EM 35Assessment of cyclodextrin extraction as a tool for determining the bioavailability of pahs in soils

PAULA CLASPER and KIRK T. SEMPLE
Dept of Environmental Science, Institute of Environmental and Natural Sciences, Lancaster University, Lancaster
LA1 4YQ

Soils were spiked with ^{14}C-radiolabelled phenanthrene at concentrations of 0, 5, 10, 25, 50, 100 mg kg^{-1} and sampled (mineralisation assay, HPCD extraction, sample oxidation) at time points of 0, 7, 14, 21, 28, 123 and 180 days. Sample oxidation showed there was no significant loss of activity within the microcosms between 0 and 28 days but at 123 days, some loss had occurred. At time 0 - 28 days, percentage mineralisation maintained a consistent level, however a reduction in percentage recovery by HPCD extraction was observed. Whilst in the early stages of mineralisation there was a divergance of extractable compared to mineralisable this trend was short lived and by 128 days the percentage of extractable phenanthrene closely resembled the percentage removal by mineralisation. HPCD extraction was comparable to mineralisation over the concentration range analysed with a ratio of HPCD to mineralisation close to 1:1 for time points T=7, 14, 21, 28, a ratio of 1.3:1 for T=0 and 1:1.06 at 123 days. HPCD extraction does provide, within the limitations of experimental design, a good assessment of microbial bioavailability as compared to the mineralisation assays. Further analyses will be carried out to determine effects on aging of phenanthrene in the presence of a co-pollutant (pyrene). These analyses will be carried out over a lower concentration range to avoid a concentration effect.

EM 36Enrichment and growth of halophilic hydrocarbon degraders

J. ASCOTT[1], C.J. BASS[1], T. McGENITY[2] and H.M. LAPPIN-SCOTT[1]
[1]Exeter University, [2]Essex University

Abstract not received

EM 37Factors influencing biofilm formation by Salmonella spp.

M.S. HAMILTON[1], G.F. MOORE[1], T.J.HUMPHREY[2] and H.M. LAPPIN-SCOTT[1]
[1]Exeter University, [2]PHL Food Research Unit, Exeter

Abstract not received

EM 38Effects of a pyrethroid-insecticide sheep dip on soil microbial activity

T. BOUCARD, R. BROOKIN, K. JONES and K.T. SEMPLE
Dept of Environmental Science, Lancaster University, Lancaster LA1 4YQ

Sheep dipping using insecticide formulations to control ectoparasites such as sheep scab, mites, ticks and lice, although no longer compulsory in the UK, is still required to comply with the Agriculture (Miscellaneous Provisions) Act 1968, and the Welfare of Animals at Market order 1990. In recent years, the use of organophosphate insecticide has been linked to human health problems and deleterious environmental effects. As a result, the use of pyrethroid insecticide in sheep dipping has increased dramatically. However, the effects of pyrethroids on health and the environment are poorly documented. As pyrethroids are highly toxic to aquatic environments, sheep dip disposal is now regulated. For disposal onto soil, the dip must be diluted with slurry or water prior to application. The aim of this study is to investigate the impact of a diluted sheep dip formulation on indigenous soil microbial activity. The formulation used was Bayticol, containing 6% flumethrin in Solvesso 200 (cont. methylnaphthalin)-solution. Soil was spiked with 4 concentrations of diluted Bayticol (0, 0.01, 0.1 and 1%), aged, and amended with ^{14}C-glucose at days 1, 7, 14, and 21. Respirometry analysis was performed to allow for estimates of ^{14}C-specific metabolic activity (rate and extent of ^{14}C-glucose mineralisation). Incorporation into microbial biomass was assessed by soil chloroform fumigation. In addition, cell numbers were estimated using the Most Probable Number method. Glucose mineralisation was significantly increased with increasing Bayticol concentrations. Cell numbers were also significantly higher in Bayticol 0.1 and 1%, compared to the control and 0.01% concentrations. Conversely, the extent of glucose incorporation into the biomass decreased with increasing concentrations. Whilst it is clear that Bayticol affects microbial communities through an increase in biomass, and thus an increase in metabolic activity, the impact of such an effect on soil function is unclear at this time.

EM 39Use of a model system to study perturbation of microbial communities in the rhizosphere

MICHAEL ROGERS[1], JUSTIN P. CLAPP[1], GARY K. ROBINSON[1], IAN P. THOMPSON[2] and PETER JEFFRIES[1]
[1]Research School of Biosciences, University of Kent at Canterbury, Canterbury, Kent CT2 7NJ, [2]Centre of Ecology and Hydrology – Oxford, Mansfield Road, Oxford OX1 3SR

Rhizosphere-associated microbial communities show far higher diversity than those found in bulk soil. Rhizosphere systems often harbour microbes beneficial to the plant, such as plant growth promoting rhizobacteria (PGPR) and a variety of antagonists to plant pathogens. Possible perturbations of these communities may be harmful to the plant and rapid monitoring of rhizosphere microbial diversity may in some situations be desirable. To this end, molecular strategies to discriminate between a range of isolates of *Pseudomonas* and *Fusarium* species were developed. Both employed the use of Polymerase Chain Reaction – Single Stranded Conformational Polymorphism (PCR-SSCP) analysis of genomic regions of the chosen organisms – for *Pseudomonas* species, the *rpoB* gene was used, and for *Fusarium*, the Internal Transcribed Spacer 2 (ITS2) region of the ribosomal DNA was used. Both regions gave species-specific SSCP profiles.

Following the development of this technology, a model system was developed in order to assess the efficacy of the technique *in situ*. Combinations of isolates of *Pseudomonas* and *Fusarium* were inoculated into rhizoboxes containing seedlings of *Lolium perenne* in the presence and absence of a chemical pollutant, 3,5-dichlorobenzoate (3,5-dCBA). RNA was extracted from a variety of root zones and subjected to RT-PCR, using appropriate primer sets. Profiles from polluted and non-polluted soils showed the extent of differences in spatial and temporal colonisation of the target groups in both treatments.

EM 40Oxidative dissolution of sulfide minerals by defined mixed populations of moderately thermophilic microorganisms

NAOKO OKIBE and BARRIE JOHNSON
School of Biological Sciences, University of Wales, Bangor LL57 2UW

The microbiologically-accelerated oxidative dissolution of sulfidic minerals such as pyrite (FeS_2) forms the basis of `biomining', an expanding area of biotechnology in which prokaryotes are used to extract metals such as gold and copper from ores and concentrates. However, the same microorganisms are also involved in generating `acid mine drainage' a severe form of water pollution affecting over 100 streams and rivers in the UK, and well as in other areas of the world. Currently, there is increased interest in using moderately (Topt 40-60°C) and extremely (Topt >60°C) thermophilic acidophiles in bioreactor systems for processing mineral ores as they are more effective at oxidising more refractile minerals, such as chalcopyrite ($CuFeS_2$) and can oxidise minerals at enhanced rates compared to mesophilic bacteria, though the microbial composition of most commercial bioleaching systems is currently poorly understood. We have sought to compare the effectiveness of a range of pure and mixed cultures of acidophilic Gram-positive and Gram-negative bacteria and an archaeon (a *Ferroplasma*-like isolate) in leaching rock-pyrite and concentrate-pyrite at 45°C in laboratory cultures. Mixed cultures were frequently more effective than the most efficient of the pure cultures (a thermotolerant *Leptospirillum ferrooxidans* strain), particularly in leaching the pyrite concentrate where there was evidence of inhibition of *L.ferrooxidans* by residual flotation chemicals. There was evidence that some moderately thermophilic bacteria which have been isolated frequently from commercial bioreactors did not contribute to net mineral dissolution in mixed culture systems.

EM 41Identification of species-specific faecal contamination in environmentally sensitive water

S. FLORINI[1], D.J. SMITH[1], M. JOHNSON[2], P. GLASS[3] and A.S. BALL[1]
[1]Dept of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, [2]Environment Agency, Cobham Road, Ipswich, [3]Anglian Water, Endurance House, Chivers Way, Histon, Cambs

Faecal contamination of environmentally sensitive waters results in the downgrading of water quality which has consequences for the economic viability of shellfisheries. In order to alleviate pressure on the shellfisheries industry and to enable the construction of appropriate management strategies the biological and geographic origin of the pollution needs to be identified. The aim of this study was to use both traditional microbiological and novel chemical analyses to identify species-specific faecal contamination impacting the Salcott Creek shellfish industry. A modified MPN assay, using microtitre plates, was used to detect and enumerate faecal coliforms and streptococci. To identify species-specific faecal pollution, sterols (chemical indicators of faecal pollution) were extracted from environmental samples and identified and quantified by GC-MS.

Results to date have identified that human contamination is the major source of organic pollution impacting Salcott Creek shellfisheries. The highest concentration of faecal coliforms were recorded at Salcott Sewage Works (SSW) outlet pipe (1.07×10^5 faecal coliforms 100 mL^{-1}). A further source of contamination was identified from a more distant sewage outlet pipe (Virley Brook). Concentrations of faecal coliforms were 25-fold lower (3.63×10^3 100 mL^{-1}) in the Virley Brook input as compared to SSW but the flow rate was significantly higher (30 L s^{-1} compared to 0.5 L s^{-1} respectively). Consequently Virley Brook is the major contributor of faecal pollution entering Salcott Creek. Identification of sterols

indicative of human pollution (e.g. coprostanol) were also identified and therefore confirmed the conclusions made from our finding using traditional microbiological assays. Further work is currently underway to establish sterol-profiles of different species (e.g. birds and livestock) which will be used to assess the relative contribution of animal faecal contamination in Salcott Creek.

EM 42 Biosorption of cadmium by free cells of *Streptomyces subrutilus*

D. TUNGTAKANPOUNG[1], I.C. HANCOCK[2] and P. J. SALLIS[1]
[1]Environmental Engineering, Dept of Civil Engineering, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, [2]Dept of Microbiology, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH

Using micro-organisms especially bacteria as biosorbents is a potential alternative method for removal heavy metal from aqueous solution. According to this research, biosorption of cadmium by free cell of *Streptomyces subrutilus* was studied with respect to effect of physico-chemical factors. Moreover, efficiency of cadmium desorption was also determined with a range of chemical extractants. The results show that, both living and non-living cells were able to uptake cadmium rapidly approximately 90% of equilibrium capacity within 15 minutes. The adsorption capacity would be decreased with reducing of pH. The minimum pH of adsorption was pH 3 for both living and non-living cells. The optimum temperature of adsorption was 30 C for living cells, however, the temperature between 20-35C did not affect adsorption capacity for non-living cells. The saturation adsorption capacity was 46.92 mg Cd/g dry weight (pH 6.0) for living cells and 36.93 mg Cd/g dry weight (pH 6.0) for non-living cells as determined by the Langmuir adsorption Isotherm. Of the tested extractants, 0.1 M HCl solution showed the highest efficiency (98%) for cadmium desorption.

EM 43 Biosorption of Cd by *Streptomyces subrutilus* immobilized on granular activated carbon

D. TUNGTAKANPOUNG[1], I.C. HANCOCK[2] and P. J. SALLIS[1]
[1]Environmental Engineering, Dept of Civil Engineering, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, [2]Dept of Microbiology, The Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4HH

Abstract not received

EM 44Naturally bioluminescent fungi – potential fungal biosensors?

HEDDA J. WEITZ[1,2], ANGELA L. BALLARD[1], COLIN D. CAMPBELL[2] and KEN KILLHAM[1]
[1]Dept of Plant and Soil Science, University of Aberdeen, Aberdeen AB24 3UU, [2]Macaulay Land Use Research Institute, Aberdeen AB15 8QH

Bioluminescence-based bacterial and yeast biosensors have been shown to be suitable tools for ecotoxicity testing. The potential of naturally bioluminescent fungi as biosensors was investigated, however little research has previously been carried out into fungal bioluminescence. Four bioluminescent fungi were selected: *Armillaria mellea*, *Mycena citricolor*, *Omphalotus olearius* and *Panellus stipticus*. The effect of culture conditions (light, pH, temperature and media composition) on bioluminescence and mycelial growth was investigated on solid media. The bioluminescence and growth of *A. mellea*, *M. citricolor*, *O. olearius* and *P. stipticus* were also characterised in shaking liquid cultures using a range of media. All fungi grew as globular mycelia but bioluminescence levels were significantly higher for *A. mellea* and *M. citricolor*. Consequently, a fungal bioassay was developed using *A. mellea* and *M. citricolor*. Bioluminescence showed a dose-response to 3,5-dichlorophenol, copper and zinc, and these results were compared with bacterial and yeast biosensors. Other compounds were also tested, including respiratory inhibitors (sodium azide and salicylhydroxamic acid). The results suggested that naturally bioluminescent fungi could potentially be used as biosensors. Work is on-going to characterise the response of the fungal biosensors to a range of compounds and to further develop the fungal bioassay.

EM 45Spatial appraisal of a hydrocarbon spill; degradation potential and toxicity assessment

CHINNY IROEGBU and GRAEME I. PATON
Dept of Plant and Soil Science, University of Aberdeen, AB24 3UU

There is a need to develop technologies to rapidly assess the impact of oil contamination both in a toxicology context and for bioremediation potential. This is particularly pertinent in the developing world where the extent of spills is often greater and technology is more restricted.

A catastrophic failure of a storage tank caused several thousand litres of diesel to be released into sub-surface soil. After eight years the hydrocarbons (H-C) had migrated to a beach and had become visible on the surface. Although remedial action was carried out to prevent further leaching of the H-C no attempt was made to clean-up the beach.

An investigation was carried out to assess:

1. The concentration and composition of H-C on the beach
2. The relative toxicity of the material
3. The bioavailability of certain key H-C fractions
4. The distribution of degraders in the environment

The methods used include gas chromatography, *lux*-based metabolic biosensors and catabolic biosensors and H-C degrader MPN techniques. These were spatially represented and analysed. It was found that there was a significant variation in the extent of the spill and as a consequent the microbial ecology was significantly impacted. While some areas will require excavation, there are promising results highlighting the opportunity for *in situ* remediation.

TUESDAY 27 MARCH 2001

0910 Applications of biocatalysis in organic synthesis

N. TURNER
University of Edinburgh

Abstract not received

0950 Preparation of enantiomerically pure sulfoxides by biocatalytic oxidation and reductive resolution

D.R. BOYD[1], H. DALTON[2], R.A. HOLT[3], O. ICHIHARA[4], H. LUCKARIFT[2], A.G. M[C]EWAN[5],G.E. ROBINSON[6] and N. SHARMA[1]
[1]School of Chemistry, The Queens University of Belfast, Belfast BT9 5AG, [2]Dept of Biological Sciences, The University of Warwick, Coventry CV4 7AL, [3]Avecia LifeScience Molecules, Belasis Avenue, Billingham, Cleveland TS23 1YN, [4]Oxford Asymmetry, 151 Milton Park, Abingdon, Oxon OX14 4JD, [6]AstraZeneca Pharmaceuticals, Hurdsfield Industrial Estate, Macclesfield SK10 2NA, [5]Dept of Microbiology, The University of Queensland, Brisbane 4072, Australia

Enantiopure sulfoxides are of interest as building blocks for the synthesis of biologically active molecules and also as ligands for chemical asymmetric catalysis. We have employed two approaches to the synthesis of sulfoxide enantiomers, asymmetric oxidation of sulfides and reductive resolution of racemic sulfoxides. Asymmetric oxidation has been achieved using toluene or naphthalene dioxygenase from Pseudomonas species which can provide either sulfoxide enantiomer according to the choice of enzyme. In addition we have demonstrated that dimethylsulfoxide reductase from a range of microorganisms is able to enantioselectively reduce racemic sulfoxides and hence provide an alternative route to enantiopure sulfoxides. A range of microoganisms expressing sulfoxide reductase activity have been isolated from soil by enrichment under anaerobic conditions in the presence of sulfoxides as terminal electron acceptors. As with the dioxygenases, either sulfoxide enantiomer can be obtained by choice of the appropriate microoganism. Initial enzyme purification studies on one of these isolates has shown there to be multiple reductase activities present. Based on crystal structure information attempts are being made to alter the substrate specificity of DMSO reductase from *Rhodobacter capsulatus* using site-directed mutagenesis.

1100 Biochemical diversity and biocatalytic potential of dehalogenating enzymes

DICK B. JANSSEN, LIXIA TANG and JEFFREY LUTJE SPELBERG
Biochemical Laboratory, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, 9747 AG Groningen, The Netherlands

A wide variety of enzymatic reactions for the dehalogenation of chlorinated substrates have been found in bacteria. The dehalogenases involved possess different structures and have diverse catalytic mechanisms. Examples are: 1) hydrolytic dehalogenation by haloalkane dehalogenases or haloacid dehalogenases, which occurs often via the formation of a covalent substrate-enzyme intermediate; 2) dehydrohalogenation of hexachlorocyclohexane, a reaction in which a *vicinal* HX pair is eliminated to form a double bond; 3) intramolecular substitution, where a *vicinal* halohydrin is converted to an epoxide; and 4) reductive dehalogenation, which requires a reducing cofactor and yields a product in which a halogen is replaced by a hydrogen. The function of these reactions is usually the bacterial utilization of halogenated compounds as a carbon- and energy source, or their use as an electron acceptor.

Biocatalysis with some dehalogenases is attractive because of their enantioselectivity. This has been established earlier for haloacid dehalogenases, and we have recently found that also haloalkane dehalogenases may be enantioselective. The most versatile dehalogenases for biocatalysis are the haloalcohol dehalogenases, which use a non-covalent mechanism in catalysis and can convert *vicinal* haloalcohols to diols and *vice versa.*

We have further explored the haloalcohol dehalogenases (also called halohydrin lyases) from strains of *Agrobacterium radiobacter* AD1 and *Mycobacterium* sp. GP1, organisms that degrade 1,3-dichloropropanol and 1,2-dibromoethane, respectively. Sequence analysis showed that the enzymes are related to the short-chain reductase-dehydrogenase superfamily of proteins. Use in biocatalysis is attractive because of the enantioselectivity, regioselectivity, and the possibility to apply in ring-opening reactions alternative nucleophiles, such as azide, cyanide, and various halides. The use of a haloalcohol dehalogenase in combination with an epoxide hydrolase improved the enantioselectivity of the dehalogenase in the preparation of optically pure halohydrins and diols. Furthermore, the application of these haloalcohol dehalogenase for the azide-dependent ring opening of styrene epoxides made it possible to prepare optically pure azidoalcohols, which can be used for further organic synthesis.

Lutje Spelberg JH, Van Hylckama Vlieg JET, Bosma T, Kellogg RM & Janssen DB (1999) A tandem enzyme reaction to produce optically active halohydrins, epoxides and diols. Tetrahedron: Asymmetry 10: 2863-2870
Van Hylckama Vlieg JET, Tang L, Lutje Spelberg JH, Smilda T, Poelarends GJ, Bosma T, van Merode AEJ, Fraaije M & Janssen DB (2001) Halohydrin dehalogenases are structurally and mechanistically related to short-chain dehydrogenases/reductases. Submitted for publication.

1140 Biocatalytic hydration, decarboxylation and methylation

JACK ROSAZZA, ALOK DHAR, KAJARI DHAR, KYEUNG SEON LEE and JANELLE TORRES Y. TORRES
Division of Medicinal and Natural Products Chemistry, Center for Biocatalysis and Bioprocessing, Oakdale Research Park, 2501 Crosspark Road Suite C100, University of Iowa, Iowa City, Iowa 52242-5000 USA -
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In Iowa, the processing of corn and soybeans releases an abundance of phenolic cinnamates, isoflavones, sterols and protein byproducts. Screening microorganisms for their capacities to alter the structures of these natural products reveals a rich array of metabolites, and a diverse range of useful biocatalytic reactions. Biotransformations of ferulic acid (FA), *p*-coumaric acid (PCA), genistein, catechin and quercetin, involve biocatalytic decarboxylations, hydroxylations, hydration, alcohol oxidation, carboxylic acid reduction and methylation. Major observed transformations of FA and PCA are decarboxylation to the corresponding 4-Vinylphenols (VP), and b-oxidation to the corresponding benzoic acids. *Nocardia* sp. NRRL 5646 efficiently transforms VP to 4-(1-hydroxyethyl)-phenol (HEP) and *p*-hydroxyacetophenone (HAP). The same organism also decarboxylates vanillic acid (VA) to form guaiacol. Mechanisms of these biotransformation reactions were probed by measuring incorporations of isotopes from $^{18}O_2$, $H_2{}^{18}O$, ^2H_2O to suggest a common quinoid-like intermediates in decarboxylation – and hydration reactions. The first bacterial catechol-O-methyltransferase from *Streptomyces griseus*, and vanillate decarboxylase from *Nocardia* were purified and characterized. Purification was enabled by creating simple

and rapid approaches to the detection of reaction products by vanillate decarboxylase (peroxidase-detection of guaiacol product), and catechol O-methyltransferase (catechol detection). Aspects of whole-cell and enzymatic biotransformations of phenolic substrates will be described.

1400 Thermostable nitrile-transforming enzymes enzymes

D.A. COWAN
Dept of Biochemistry, University College London

Mesophilic nitrile transforming enzymes are widely dispersed in the Bacteria and lower orders of the eukaryotic kingdom. Two distinct enzyme systems, a nitrilase catalysing the direct conversion of nitriles to carboxylic acids and separate but co-transcribed nitrile hydratase and amidase activities, are now well known. Two related classes of nitrile hydratase have been identified - containing either a cobalt(II) or iron(III) cofactor. Purified mesophilic nitrile degrading enzymes are typically thermolabile in buffered solution, rarely withstanding exposure to temperatures above 50°C without rapid inactivation. However, operational thermostability is often increased by addition of aliphatic acids or by use of immobilised whole cells. Low molecular stability has frequently been cited as a reason for the limited industrial application of "nitrilases"; Such statements notwithstanding these enzymes have been successfully applied for more than a decade to the multi-kiloton production of acrylamide and more recently to the smaller scale production of nicotinic acid.

Until recently, no examples of nitrile transforming enzymes from thermophilic micro-organisms had been reported. However, in the course of the past 5 years we have isolated and carried detailed structural and functional characterisation of both nitrile hydratase/amidase and nitrilase enzymes from moderately thermophilic bacteria. While showing the higher degree of thermostability expected of thermophile-derived enzymes, both the structural and functional characteristics of these enzymes are closely related to their mesophilic homologues. The typical pattern of specificity is retained, where the nitrile hydratases are apparently restricted to aliphatic substrates while the nitrilases show broad specificity with a preference for aromatic or heterocyclic nitriles. However, there is evidence that aromatic nitriles are high affinity competitive inhibitors of thermophilic nitrile hydratases, suggesting that both the mechanism of inhibition and the substrate specificity may be amenable to site specific mutagenesis.

We have cloned and expressed a single thermophilic nitrile hydratase gene, and investigated the applications of both thermostable nitrilases and nitrile hydratases in the biotransformation of acrylic acid and 3-cyanopyridine. Good operational performance was observed using immobilised cells in a plug-flow configuration at 50°C.

1440 New lyases for C-C bond formation

M. MULLER, M. POHL, A. LIESE and C. WANDREY
Institute of Biotechnology, Julich, Germany

Abstract not received

1500 Food ingredients from bioconversions: a natural challenge

JOHN T. SIME
Zylepsis Ltd, 6 Highpoint, Henwood Business Estate, Ashford, Kent TN24 8DH - **Error! Bookmark not defined.**, **Error! Bookmark not defined.**

The consumer demand for natural products and natural ingredients in formulated food products provides a particular place for biotransformations in generating such materials. There are particular instances where the employment of bioconversions provides the only feasible method of production.

Starting from bulk, low cost natural materials the challenge is to produce high value end products whilst complying with the regulatory restrictions compatible with the natural label. Using non-hydrolytic enzyme transformations on such material in multi-step bioconversions raises challenging operational considerations. Demonstration of scientific feasibility has to be translated into a production process to become commercially significant.

Procedures employing a mixture of crop processing and bioconversions into an integrated process are stretching the current capabilities of those involved in this area of technology. These obstacles are, however, being addressed and overcome by companies determined to succeed in this industry sector.

1600 Cofactor regeneration using a soluble pyridine nucleotide transhydrogenase for the biological production of hydromorphone

BIRGITTE BOONSTRA, DEBORAH A. RATHBONE and NEIL C. BRUCE
Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT

The morphine alkaloids, of which morphine, codeine and thebaine are extracted from the opium poppy *Papaver somniferum*, provide some of the most important painkilling drugs in clinical use. Synthetic routes for many derivatives of the naturally occurring morphine alkaloids are unsatisfactory. Like many bioactive compounds, these alkaloids possess a variety of different functional groups that generally require protection during chemical transformation ultimately resulting in poor yields. Biotransformations can offer advantages over chemical routes and, to this end, recombinant hybrid pathways for the biological production of important "semisynthetic" opiate drugs have been engineered.

The metabolic activities of microorganisms capable of transforming morphine and codeine to several important therapeutic compounds have been exploited for the transformation of morphine to the semisynthetic opiate drug hydromorphone, a powerful analgesic seven times more potent than morphine. To circumvent the use of expensive pyridine nucleotide cofactors in cell-free systems recombinant strains of *E. coli* that express the morphine alkaloid transforming enzymes were constructed. However, the recombinant biotransformation system suffered from low yields and accumulation of unwanted side product partly as a consequence of the cofactor dependency of the transforming enzymes. We have addressed the problem of the transfer of reducing equivalents between the nicotinamide cofactors NAD and NADP by introducing a soluble pyridine nucleotide transhydrogenase into the recombinant biotransformation system. By applying this enzyme to the whole cell biotransformation system cofactor cycling was facilitated and an improved biocatalyst with an extended lifetime was generated. The results demonstrate the usefulness of the soluble transhydrogenase as a tool in biocatalysis and metabolic engineering.

1620 Selective oxygenation reactions: new dioxygenase options

D.R. BOYD[1], N.D. SHARMA[1], D.T. GIBSON[2] and H. DALTON[3]
[1]School of Chemistry, The Queen's University of Belfast, Belfast BT 5AG, [2]Dept of Biological Sciences, University of Warwick, Coventry, CV4 7AL, [3]Dept of Microbiology, The University of Iowa, Iowa City, Iowa 52242, USA

Wild type, mutant and recombinant strains of bacteria containing dioxygenase enzymes have been found to catalyse a remarkable range of oxidation reactions. These oxidations include regio- and stereo-selective mono-, and poly-hydroxylation reactions of alkenes and arenes, sulfoxidation and desaturation. Representative examples of the latter reaction types are provided using mono- and poly-cyclic arenes and heteroarenes as substrates. The effect of minor changes in substrate or dioxygenase structure on the production of unusual regioisomers and enantiomers will be discussed. Recent examples of multistep (tandem) biotransformation reactions on arene substrates

involving dioxygenases will be presented. Factors which allow the outcome of dioxgenase-catalysed reactions of arene substrates to be predicted are summarized.

WEDNESDAY 28 MARCH 2001

0900 Biocatalysis for the preparation of fine chemicals

A. KIENER
LONZA Ltd, Visp, Switzerland

Abstract not received

0940 Bioengineering of yeast for self-sufficient steroids production

D. POMPON
CNRS, Centre de Genetique Moleculaire, Gif-sur-Yvette, France

Abstract not received

1050 The biological production of 1,3-propanediol from glucose

G. WHITED
Genencor International, Palo Alto, USA

Abstract not received

1130 Expanding the substrate range of *E. coli* transketolase by site directed mutagenesis

J. WARD (University College London)

Abstract not received

1150 Exploring protein function landscapes by molecular breeding

J. MINSHULL and P. LONGCHAMP
Maxigen Inc, Santa Clara, USA

Abstract not received

1430 Combinatorial biocatalysis for new compound and new process discovery

JONATHAN S. DORDICK
Dept of Chemical Engineering, Rensselaer Polytechnic Institute, Troy, NY 12180

In a perfect world, there would be no disease, ailments would be treated by simple and readily available compounds, and materials of use in the chemical, pharmaceutical, and food industries would be easy to conceive and synthesize. Nevertheless, nothing is perfect and as a result, we must continue to develop new technologies for the discovery of new chemical agents, new catalysts, new polymers and materials, etc. Nature owes its unparalleled structural and functional diversity to the power of enzymes and multi-enzyme pathways that comprise the synthetic machinery of biological systems. Mankind has only been able to tap into a small part of this biocatalytic repertoire, yet this has resulted in a vast array of natural products for use as pharmaceuticals, agrochemicals, chemical intermediates, and biomaterials. Nevertheless, a significantly larger and more diverse universe of natural compounds, as well as the enzymes and metabolic pathways that generate such molecules, remains untapped. New and high-throughput biocatalytic technologies will be necessary to gain access to nature's "warehouse" of structures and functions, and to be able to manipulate the synthesis of these molecules to yield novel compounds and materials. Critical to this paradigm is the need to develop high-throughput techniques to explore biocatalytic diversity, both natural and synthetic that will likely speed the inclusion of biocatalysis in discovery methodologies for use in the pharmaceutical, chemical, and agrochemical industries.

We are using biocatalysis as a discovery tool in the development of new biologically active compounds as well as new polymeric materials, and using two strategies: combinatorial biocatalysis and microarray/microfluidics bioprocessing. In this talk, we will focus on these two strategies. In the former, we have developed techniques to use a wide variety of enzymes under a multitude of processing conditions to generate libraries of new molecules, ranging from bioactive and polymeric. In the latter, we have fabricated enzyme-containing chips that enable high-throughput biocatalysis to be used along side high-throughput chemical synthesis and compound screening. Both technologies should enhance the ability of biocatalysis to be a valuable tool in discovery.

1510 Process evaluation and scale-up of whole cell Baeyer-Villiger catalysts

JOHN M. WOODLEY
The Advanced Centre for Biochemical Engineering, Dept of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE

The development of new biocatalytic conversions of use to industry (eg carbon-carbon bond synthesis and redox reactions) requires not only proof of concept and development of the biocatalyst but also the development of the necessary underpinning biochemical engineering. At UCL for the past decade, we have been researching techniques to assist in the rapid design, operation and scale-up of biocatalytic reactions. In order to illustrate our thinking, in this talk I will focus on the application of these techniques to Baeyer-Villiger oxidations catalysed by cyclohexanone monooxygenase to synthesise optically pure lactones. Work will be described on the development of a process using a recombinant whole cell biocatalyst, *Escherichia coli* TOP10 [pQR239], expressing cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871. The process was characterised at a small scale to provide the necessary insight to scale-up a fed-batch process to oxidise bicyclo[3.2.1]hept-6-en-one to its corresponding regio isomeric lactones, (-) 1(S), 5(R) 2-oxabicyclo[3.3.0]oct-6-en-3-one and (-) 1(R), 5(S) 3-oxabicyclo[3.3.0]oct-6-en-2-one. This was operated in a 75 L reactor at pilot plant scale. Process bottlenecks and directions for future process studies will also be addressed.

1550 Enzymatic and microbial production of pharmaceutical important diols

C. WANDREY, M. MÜLLER, A. LIESE and W. HUMMEL
Institute of Biotechnology 2, Research Center Jülich, D-52425 Jülich, Germany

Optically active diols are found frequently in natural products, pharmaceuticals, and ligands used in homogeneous catalysts. Biocatalysis was successfully employed for the production of chiral 1,2-, 1,3- and 1,4-diols. (*S*,*S*)-2,5-Hexandiol, an important bidentate ligand for homogeneous catalysis, was synthesized in 10 kg scale employing whole cell biotransformation.

All four stereoisomers of *tert*-butyl 6-chloro-3,5-dihydroxyhexanoate in high optical purity were obtained in a bioorganic synthesis using isolated enzymes, whole cells and classical organic chemistry. These building blocks play an important role, e.g. in the synthesis of HMG-CoA reductase inhibitors.

All diols are obtained from the corresponding dions. Formate, *iso*-propanol and glucose is used as hydrogen source for the bioreductions.

POSTERS

FbPbmg 01 Glutamine synthetase activity in *Micromonospora Echinospora*

PAUL A. HOSKISSON, GLYN HOBBS and GEORGE P. SHARPLES
School of Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF

Two thirds of the known aminoglycoside antibiotics are produced by actinomycetes other than *Streptomyces*, with a significant number of these being isolated from the genus *Micromonospora*. Despite the industrial potential of *Micromonospora* species, studies of their physiology are few. Our work has focused on nitrogen metabolism in the gentamicin producer, *Micromonospora echinospora*. Glutamine synthetase (GS), is the central enzyme in ammonium assimilation, which can be regulated at the gene

and enzyme level. In the present study, we have investigated the activity of GS under carbon and nitrogen limiting conditions, in continuous and batch culture. It was found that alanine as a sole nitrogen source resulted in the highest GS activity. The addition of 0.2 % (w/v) ammonium chloride resulted in an 80 % reduction in GS activity within 4 minutes, suggesting post-translational adenylation plays a part in regulation of nitrogen metabolism in *Micromonospora*.

FbPbmg 02 Differential protease production by *Micromonospora Echinospora* in carbon and nitrogen-limiting cultures

PAUL A. HOSKISSON, GLYN HOBBS and GEORGE P. SHARPLES
School of Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool L3 3AF

Members of the genus *Micromonospora* exhibit complex developmental cycles, differentiating both morphologically and physiologically. The physiology of the genus is poorly understood when compared with other actinomycete genera such as *Streptomyces*. This particular study has focused on the production of proteolytic enzymes in the gentamicin producer, *Micromonospora echinospora*. The production of extracellular proteolytic enzymes in *M. echinospora* was studied in liquid minimal medium, under conditions stoichiometrically limiting for carbon and nitrogen. Purification of the proteases by affinity chromatography, and subsequent electrophoresis demonstrated three proteases expressed under carbon-limiting conditions. Nitrogen limitation, however, revealed the expression of up to 10 proteases.

FbPbmg 03 The production of novel sordarin analogues by biotransformation

RICHARD M. HALL, CAROL A. JONES, PAUL STEAD, PHILIP J. SIDEBOTTOM and NICK L. TAYLOR
Glaxo Wellcome Research and Development, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY

Opportunistic fungal infections are increasingly common in severely immunocomprimised patients especially following cancer chemotherapy, bone marrow or organ transplantation and HIV infection. The prognosis for these patients is typically very poor. Consequently, the search for novel anti-fungal compounds displaying new modes of action and improved pharmacological characteristics has intensified across the pharmaceutical industry.

Sordarin, a diterpene glycoside produced by the fungus *Sordaria araneosa*, is a key intermediate in the synthesis of novel anti-fungal agents. These compounds are highly selective inhibitors of fungal protein biosynthesis targeting elongation factor 2 (EF-2) and the ribosomal P-protein. Sordarin derivatives have been shown to inhibit most fungal pathogens known to affect immunocomprimised patients. These include *Candida albicans* and a number of non-albicans species. Excellent activity has also been observed against the opportunistic pathogen *Cryptococcus neoformans* and also *Pneumocystis carinii* the causal agent of pneumonia. It has also been demonstrated that sordarin derivatives have potent fungicidal activity against important dimorphic endemic fungal pathogens such as *Histoplasm capsulatum, Blastomyces dermatitidis, Paracoccidioides brasiliensis* and *Coccidioides immitis.* However, two important pathogens namely *Aspergillus fumigatus* and *Candida krusei* are both essentially resistant to these compounds

Using a whole-cell microbial biotransformation approach, both sordarin and the closely related aglycone sordaricin were biotransformed by a range of organisms yielding a number of novel modified derivatives. Biotransformations ranged from simple esterifications to reduction, oxidation and demethylation. A number of organisms were scaled up to support both structure-activity studies and further chemical derivatisation. Details of this work will be presented.

FbPbmg 04 The Biotransformation of nitriles by *Rhodococcus rhodochrous* strain LL100-21 when immobilised and in the presence of a DC field

ROBERTA MUSTACCHI, SIMON JACKMAN and CHRIS KNOWLES
The OCEB, NERC Centre of Ecology and Hydrology-Oxford, and the Dept of Engineering Science, University of Oxford

Rhodococcus rhodochrous LL100-21 is a bacterium that is capable of the specific biotransformation of nitriles into the corresponding carboxylic acids and ammonia. In this study pure cultures of *R.rhodochrous* were used for the biotransformations of nitriles in the presence of a DC field. The application of an electric field to growing cultures caused a change in the morphology during cell growth. Rhodococci normally undergo a change of shape (from cocci to rods) during the exponential phase of growth. In the presence of direct current the cells remained as cocci throughout the entire growth curve. Membrane fatty acid analysis showed an increased level of fatty acid variability during growth with the current. Bacterial viability was also drastically affected by the presence of the current. In the case of cells grown in absence of current the increase of viable and total cell count is directly proportional. When DC was applied to the cells a death rate of 300 CFU/ml/hr was observed. The decrease in the number of viable cells stopped when a plateau of low, constant viability had been reached. Despite the fact that the use of an electric field caused physiological changes to the cells and decreased the overall viability of the culture, no significant effect was noticed on the biotransformation rate. The effect of whole cell immobilisation to the biotransformation of nitriles is also being investigated.

FbPbmg 05 Molecular genetics of polysaccharide breakdown by *Eubacterium* spp. from the human colon - potential for pro / prebiotics

ALAN G. RAMSAY, K.P. SCOTT, H.J. FLINT and G.W. GOODAY
Rowett Research Institute, Dept of Gut Microbiology and Immunology, Greenburn Road, Bucksburn, Aberdeen AB2 9SB

Dietary polysaccharides that are not digested in the small intestine provide a major source of energy for the commensal obligately anaerobic bacteria resident in the large intestine. Since some of these bacteria contribute to healthy gut function directly by producing nutrients required by the colonic mucosa and also affect pathogen exclusion, elucidating methods to manipulate the dietary balance are of great interest. Our group have recently shown that a large proportion of butyrate producing anaerobes isolated from faecal samples correspond phylogenetically to previously unidentified bacteria but are closely related to *Eubacterium* spp., and fall in cluster XIVa of the *Clostridium* subphylum of low G+C% gram-positive bacteria. The role of butyrate in the metabolism and development of a healthy colonic epithelium and thus in the prevention of cancer and ulcerative colitis is well documented. The ability of chosen isolates to utilise starch, a known butyrogenic polysaccharide, has been investigated. The results indicate a strong preference for starch with a high amylopectin content with these colonic isolates. SDS-PAGE zymograms allowed a clear identification of different active amylase bands in bacterial extracts, showing uncommonly high molecular masses (150 - 200kDa). This work is aims to identify the enzymes involved in starch degradation, their organisation and regulation. This work also describes for the first time, the survival of chosen *Eubacterium*-like strains under simulated colonic conditions.

FbPbmg 06 Engineering recombinant biocatalysts for the N-demethylation of morphine alkaloids

ERIK R. GIMPEL and NEIL C. BRUCE
Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT

Morphine alkaloids have three important pharmacological properties, anti-tussive, analgesic and narcotic antagonist. The plant extracted alkaloids can be chemically transformed to a range of semi-synthetic compounds. A pivotal intermediate in the sythesis of narcotic antagonists requires an *N*-demethylation of the alkaloid, which is a difficult and inefficient chemical reaction. Hence enzymatic catalysis of the transformation is an attractive target.
N-demethylation of codeine has been reported in *Streptomyces griseus*, *Cunninghamella echinulata* and in the human liver, and in all these systems it has been shown that a cytochrome P450 is the catalytic enzyme.

Cytochrome P450 genes have been selected and isolated, overexpressed with and without their associated ferredoxins in *E. coli* and *S. lividans*. *N*-demethylase activity was assayed in whole cells and the recombinant enzymes characterised.

We are able to introduce *N*-demethylase activity into various organisms and this will be further used in engineering biological pathways for the synthesis of valuable compounds.

FbPbmg 07 Biological generation of oxyfunctionalised intermediates for semi-synthetic opiate synthesis

CAMPBELL TANG, ADAM WALKER and NEIL C. BRUCE
Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT

The morphine alkaloids include many potent and widely used analgesics, antitussives and narcotic antagonists, with semi-synthetic derivatives constantly being developed to further improve their properties. Difficulties are often encountered during chemical synthesis, making the use of biotransformations for the production of semi-synthetic derivatives a significant area of research.

We are concerned with the generation of chemically difficult to obtain oxyfunctionalised intermediates, which can be used as molecular platforms upon which various chemical modifications can be performed, thereby producing valuable semi-synthetic opiate drugs.

Various strains of two organisms, *Pseudomonas putida* M10 and *Pseudomonas testosteroni*, have been tested and compared in whole cell and crude extract biotransformations, for their ability to hydroxylate morphine alkaloids, particularly in the C14 position. Presently, maximum turnovers observed are approximately 30%. The identification and characterization of the hydroxylase is currently in progress.

FbPbmg 08 The *nag* genes converting gentisate to central metabolites in *Ralstonia* sp. strain U2

NING-YI ZHOU, SERGIO L. FUENMAYOR and PETER A. WILLIAMS
School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

Ralstonia sp. strain U2 metabolises naphthalene via gentisate (2,5-dihydroxybenzoate) to central metabolites. The genes are located on a single operon *nagAaGHAbAcAdBFCQEDJIKLMN*. The upstream genes as far as *nagD* are very similar in sequence and order to the homologous classical *nah* genes on catabolic plasmid NAH7 and encode the conversion of naphthalene to salicylate but with the additional insertion of *nagGH* (see adjacent poster). Downstream of *nagD*, the genes encode the catabolism of gentisate. All the genes have been cloned into expression vectors and expressed singly or in combinations. Three of them only have been shown to be necessary for gentisate catabolism. NagI is a gentisate 1,2-dioxygenase, converting gentisate to maleylpyruvate, NagL is a reduced glutathione-dependent maleylpyruvate isomerase, effecting a *cis-trans* isomerisation to fumarylpyruvate and NagK is a fumarylpyruvate hydrolase, which cleaves a carbon-carbon bond producing stoichiometric quantities of fumarate and pyruvate. The other adjacent genes cluster appear to play no part in the enzymatic steps. NagJ is a glutathione S-transferase homologue, similar to others of unknown function found in aromatic catabolic gene clusters, and NagM and NagN are homologues of each other and bear no relationship with other proteins with identified functions in the databanks.

FbPbmg 09 Salicylate 5-hydroxylase from *Ralstonia* sp. strain U2: a novel monooxygenase closely related to aromatic hydrocarbon dioxygenases

NING-YI ZHOU and PETER A. WILLIAMS
School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

Ralstonia sp. strain U2 metabolises naphthalene via gentisate (2,5-dihydroxybenzoate) to central metabolites. At the 5' end of a 18-gene operon encoding the complete *nag* genes is a gene cluster (*nagAaGHAbAcAd*) encoding the naphthalene dioxygenase (*nagAcAd*), its electron transport chain of ferredoxin reductase (*nagAa*) and ferredoxin (*nagAb*) plus a putative 5-salicylate hydroxylase (*nagGH*) which converts salicylate (2-hydroxybenzoate) to gentisate. NagG shows strong homology to NagAc and other ISP proteins involved in the conversion of aromatic hydrocarbons to *cis*-dihydrodiols.

We have separately expressed the salicylate 5-hydroxylase genes (*nagGH*), the reductase (*nagAa*) and the ferridoxin (*nagAb*) in high expression vectors. All three cell-free extracts were required for the salicylate 5-hydroxylase activity in the presence of ferrous and NADH and no activity was detected in the absence of NagAa or NagAb. Reduction of any one component in the mixture made it a rate-limiting step thus demonstrating the essentiality of all components. The optima pH, kinetics and substrate specificity of this enzyme have been determined.

This represents a unique scenario where NagG and NagH, the structural subunits of salicylate 5-hydroxylase, a monooxygenase, are linked to an electron transport chain consisting of NagAb (ferredoxin) and NagAa (ferredoxin reductase), which are shared with NagAcAd, the structural subunits of naphthalene dioxygenase.

FbPbmg 10 The 4-nitrobenzoate genes and their enzymes from *Pseudomonas putida* strain TW3

MICHELLE A. HUGHES and PETER A. WILLIAMS
School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

Pseudomonas putida strain TW3 is able to utilize 4-nitrotoluene (a by-product of the explosives industry) and 4-nitrobenzoate as the sole sources of carbon and nitrogen. The nitro group of 4-nitrotoluene is retained during the sequential oxidation of the methyl group to form 4-nitrobenzoate via a route analogous to the upper pathway of the TOL plasmids. The further catabolism of 4-nitrobenzoate occurs via the hydroxylaminobenzoate pathway to the ring cleavage substrate 3,4-dihydroxybenzoate (protocatechuate) with the release of ammonia.

We have cloned the genes encoding the 4-nitrobenzoate catabolic enzymes from TW3 by mobilising a cosmid library of TW3 DNA into *P. putida* strain PaW340 and selecting for transconjugants able to grow on 4-nitrobenzoate: this was achieved by a 6 kbp fragment of TW3 DNA carrying a cluster of five genes. Only two of these encoded the enzymes converting 4-nitrobenzoate to protocatechuate, designated PnbA and PnbB. Their function has been demonstrated by creating knockouts, by expressing the genes in *E. coli* and by biochemical assays. PnbA encodes a 4-nitrobenzoate reductase responsible for the direct reduction of 4-nitrobenzoate to 4-hydroxylaminobenzoate. The further conversion of 4-hydroxylaminobenzoate to protocatechuate and ammonium is carried out by PnbB, a 4-hydroxylaminobenzoate lyase enzyme. Using purified proteins we have determined the pH optima and the substrate specificities of these enzymes.

FbPbmg 11 Site-directed mutagenesis of chromosomal genes in the naturally competent *Acinetobacter* sp. ADP1 using overlap extension PCR mutagenesis and a *sacB*-Km cassette

RHEINALLT M. JONES and PETER A. WILLIAMS

School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

Acinetobacter sp. ADP1 is a naturally transformable bacterium much studied as a model organism for the catabolism of aromatic compounds. It has a network of regulated catabolic operons that use a wide variety of natural aromatics derived from the biodegradation of lignin and suberin.

We have developed a method of site-directed mutagenesis of chromosomal genes in ADP1 making use of its natural transformation. A cassette containing a kanamycin resistance gene and the *sacB* gene is inserted into the region to be mutated: the presence of *sacB* inhibits growth of the bacteria in the presence of sucrose by causing synthesis of toxic oligosaccharides. Using the natural competency of ADP1 the inserted cassette is replaced by DNA fragments of chromosomal DNA modified by overlap extension PCR mutagenesis to carry specific designed mutations: mutant transformants are selected by acquisition of the ability to grow in the presence of sucrose. This method provides a straightforward procedure for mutation by a frameshift, deletion, insertion or substitution of one or more bases in genes located in their natural chromosomal location.

We have successfully used this method to mutate ADP1 in the putative regulatory DNA upstream of *salA*, a gene encoding an esterase which catalyses the hydrolysis of alkyl salicylates to an alcohol and salicylic acid.

FbPbmg 12 Dioxygenase catalysed oxidation of Thiophene compounds by *Pseudomonas putida*

SUZANNE L BALLINGER[1], HOWARD DALTON[1], NIMAL GUNARATNE[2], NARAIN D. SHARMA[2] and DEREK R. BOYD[2]
[1] Dept of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry, [2] School of Chemistry, Queens University of Belfast, Belfast

Aerobic microorganisms such as *Pseudomonas putida* contain a toluene dioxygenase enzyme which catalyses the dioxygenation of arene molecules to produce a wide range of enantiopure *cis*-dihydrodiols. *P. putida* UV4 is a mutant strain which lacks a dihydrodiol-dehydrogenase but is constitutive for toluene dioxygenase expression. Biotransformations catalysed by the dioxygenase enzyme system have been shown to produce 2,3-*cis*-dihydrodiols from monosubstituted arene substrates. This enzymatic oxidation system is very versatile and without parallel in organic synthesis.

It has recently been demonstrated that the dioxygenase system can also be used to yield unique enantiopure sulfoxides with high enantiomeric excess values. Results will be presented which demonstrate the effect of substituted functional groups upon the biocatalytic oxidation of thiophenes. For example 2-substituted thiophenes yield novel sulfoxide intermediates. In contrast, 3-substituted thiophenes gave optically active interconverting *cis/trans*-dihydrodiol intermediates without the production of sulfoxide products. Further modification of the 2-substituted thiophene sulfoxides (thiophene oxides) via a spontaneous Diels-Alder addition reaction yields sulfoxide dimers.

FbPbmg 13 Purification and stereochemical properties of DMSO reductases

HEATHER R. LUCKARIFT and HOWARD DALTON
Dept of Biological Sciences, University of Warwick, Gibbet Hill Road, Coventry

During the anaerobic growth of organisms such as *Escherichia coli, Proteus* spp. and *Rhodobacter* spp., C_1 compounds such as DMSO (dimethylsulfoxide) can function as an alternative electron acceptor to oxygen and is reduced to DMS (dimethylsulfide) by the enzyme DMSO reductase. The DMSO reductase enzyme is also capable of stereoselective reduction of chiral sulfoxides to the corresponding sulfides.

We are currently investigating the ability of the DMSO reductase enzymes to perform stereoselective kinetic resolution of sulfoxide substrates. The transformation of racemic sulfoxides by these enzymes results in the selective removal of one enantiomer which is converted to the corresponding sulfide leaving the other enantiomer intact. The enantioselective reduction of racemic sulfoxides can be exploited for the preparation of optically pure sulfoxides, which have great potential as chiral synthons.

We have isolated bacteria that are capable of resolving sulfoxide substrates to give configurations that are strain specific, thereby giving us access to both enantiomers depending on the strain used and providing the opportunity to compare the mechanisms of kinetic resolution within these organisms.

We are presently investigating the specificity and diversity of purified enzymes from these isolates. Data will be presented on the purification and stereoselectivity of these proteins and if available, the X-ray crystal structure of enzymes with differing enantioselectivity.

FbPbmg 14 Control of specificity in alkene monooxygenases

SARAH ASKEW and DAVID LEAK
Imperial College of Science Technology & Medicine, London

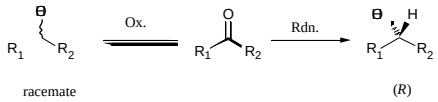
Abstract not received

FbPbmg 15 Whole cell microbial deracemization of secondary alcohols

GRAHAM ALLAN and ANDREW J. CARNELL
Dept of Chemistry, The University of Liverpool, Liverpool

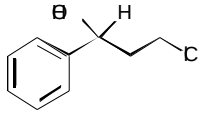
Enantiomerically pure secondary alcohols are important as chiral auxiliaries and as intermediates in the synthesis of molecules of pharmaceutical importance.[1] Current methodologies for the synthesis of enantiomerically pure secondary alcohols include microbial or enzymatic reduction of the corresponding ketones,[2] the kinetic resolution *via* esterification or hydrolysis using lipase enzymes[3] and the dynamic kinetic resolution using a coupled ruthenium/enzyme system.[4]

We would like to report the whole cell microbial deracemization of a range of secondary alcohols with liquid cultures of the bacteria *Sphingimonas Paucimobilis*.



R_1= aryl, cyclohexyl
R_2= alkyl

Typically yields of 70-90% of the (*R*)-alcohol of e.e. 90-99% are obtained with a residual amount of the corresponding ketone 10-20% being observed. Deracemizations on a 50 mg scale are reported for 15 alcohols with varying R_1 and R_2 and the scale up (250 mg) reported for 1-phenylethanol and 1-thienylethanol. The deracemization of a key intermediate in the synthesis of the antidepressant, Fluoexitine (Prozac®),[5] is also reported.



Attempts to elucidate the mechanism of the deracemization and to identify the nature of the enzymes responsible using a cell free protocol are reported.

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FbPbmg 16 Novel uses of lipoxygenases

PAUL HOUGH[1], BRIAN ROBINSON[1], ROD CASEY[2], RICHARD HUGHES[2] and PHIL TAYLOR[3]
[1]School of Chemical Sciences, UEA, [2]John Innes Institute, Norwich, [3]ICI Paints, Slough

We have used lipoxygenases from a range of sources in studies of some reactions in microemulsions relevant to the reaction of oxygen and linoleic acid.Soybean lipoxygenase was found to be a particularly effective catalyst for this reaction.

A range of water-in-oil microemulsion dispersions have been investigated where the charge on the surfactant was systematically varied. The results of these experiments will be discussed.

FbPbmg 17 Bacterial biodegrading of arsenobetaine in monoseptic culture

A.W. RITCHIE, N. MOLENAT, J.S EDMONDS, C.F. HARRINGTON and R.O. JENKINS
Dept of Biological Sciences, De Montfort University, The Gateway, Leicester LE1 9BH

Arsenobetaine [$(CH_3)_3As^+CH_2COO^-$; AB] is an arsenic-containing non-toxic organic compound that is considered to be ubiquitous in the marine environment and is generally regarded as a key compound in environmental arsenic cycling. Whereas biodegradation of AB under aerobic conditions by undefined mixed-cultures and by defined bacterial consortia has been reported previously, there are no reports of AB biodegradation by monoseptic cultures of bacteria.

We present evidence for AB biodegradation, involving cleavage of the C-As bond, by isolates from the marine environment when grown in monoseptic culture. ^1H NMR and HPLC-AAS were used to screen for AB biodegrading capability in aerobic enrichment cultures involving inocula from the marine environment. Trimethylarsine oxide (TMAO), dimethylarsinic acid (DMAA) and methylarsonic acid (MAA) - potential breakdown products of AB - were detected after microbial enrichment of mussel extract on mineral salts medium containing glycinebetaine (the N containing analogue of AB) as main carbon and nitrogen source. Isolates from positive enrichment cultures were tested for AB biodegradation in monoseptic culture using HPLC-hydride generation-atomic fluorescence spectrometry. DMAA was detected after 21-days incubation in monseptic cultures of *Brevundimonas versicularis*, *Aeromonas salmonicida*, *Flavobacterium* sp and *Pseudomonas* sp. These data suggest that the isolates degrade AB to DMAA via a route not involving TMAO, possibly through demethylation of AB prior to C-As bond cleavage, i.e. involving dimethylarsinoyl acetic acid as an intermediate. Detection of TMAO in the undefined mixed cultures and its apparent absence in monoseptic cultures suggests that two pathways of AB biodegradation exist, located in different bacterial species.

FbPbmg 18 A microbial basis for biomethylation of antimony in anaerobic environments

LOUISE M. SMITH and RICHARD O. JENKINS
Dept of Biological Sciences, De Montfort University, The Gateway Leicester LE1 9BH

Antimony (Sb) is found naturally in the environment in trace quantities, but widespread industrial utilisation has redistributed this element so that it intermingles with biological food chains at high concentrations. Whereas the biotransformations of arsenic - a Group 15 cogener of antimony - have been intensively studies, those governing the mobility and toxicity of antimony are poorly understood. Methylantimony species have been found in various natural and man-made environments, while certain aerobic fungi and mixed cultures of anaerobic bacteria are known to biomethylate inorganic antimony.

We present evidence for antimony biomethylation by monoseptic cultures of clostridia. Gas chromatography - mass spectrometry (GC-MS) was used to screen for antimony biovolatilisation capability in various soils by anaerobic enrichment culture. Trimethylantimony (TMA) was found in the headspace of cultures designed to promote growth of clostridia. GC-MS profiles of headspace gases of mixed cultures able to generate TMA was used to select *Clostridium* spp for testing for antimony biomethylation capability, by hydride generation - gas chromatography - atomic absorption spectrophotometry. Methylantimony species were detected in the culture medium of monoseptic cultures of *C. acetobutylicum*, *C. butyricum* and *C.cochlearium*. Two clostridial isolates from soil enrichment culture were also shown to biomethytlate inorganic antimony in monoseptic culture. Antimony biomethylation by clostridia, which are known to participate in the multistage process of methanogenesis, may account for the presence of volatile antimony in biogas from landfill sites. The relative quantities of mono-, di- and trimethylantimony species produced illuminates the mechanism of antimony biomethylation in these bacteria.

TUESDAY 27 MARCH 2001

0905 Use of 3-D structure information to identify enzyme inhibitors

T. FISCHMANN
Schering Plough Research Institute, Kenilworth, USA

In bacteria newly synthesized peptides are formylated at the N-terminus. Maturation of the nascent protein requires removal of the formyl group. Deformylation is accomplished by peptide deformylase (PDF). This enzyme is essential for bacterial survival, but is absent in eukaryotic cells. Thus PDF is a promising target for new antibiotics. Using an improved version of a published purification protocol, we have been able to grow crystals of *E. coli* and *Staph. aureus* PDF which diffract at very high resolution, up to 1.4 Å. These crystals contain the biologically relevant iron-containing form of deformylase. On the basis of the structural similarity between PDF and matrix metalloproteases, we have predicted that a new class of inhibitors could be derived with hydroxymate as the iron-chelating group. This observation was confirmed by the discovery of such an inhibitor, actinonin, by high-throughput screening. The X-ray structure of the *E. coli* PDF-actinonin complex at high resolution (1.5 Å) was the initial template for a structure-based drug-design program of PDF inhibitors. A large number of compounds have been inferred with nanomolar activities and potential wide-range applications. These compounds present variations in the iron-chelating group and at the P1', P2' and P3' sites. Overall the X-ray three-dimensional structures of eighteen *E. coli* PDF-inhibitor complexes and the ligand-free structure of *E. coli* and *Staph. aureus* PDF have been determined. The SAR data will be discussed in light of these structures.

0950 3-D structure analysis coupled with high throughput screening

PATRICK DORR[1], JO COTTRELL[1], TANYA PARKINSON[1], DAVID WINPENNY[1], RICHARD BAZIN[2], COLIN GROOM[2], ANDREW HOPKINS[2], MARGARET O'GARA[2], ROBERT MOORE[2], VICKY STEPHENSON[3], ANDREW BELL[4], DUNCAN ARMOUR[4], MARTIN EDWARDS[4], MARK KEMP[4], ANTHONY WOOD[4], CHRIS BULAWA[5] and JIM COOK[5]
[1]Discovery Biology, [2]Molecular Informatics, Structure and Design, [3]Physical Sciences, [4]Discovery Chemistry, Pfizer Global R&D, Sandwich, Kent CT13 9NJ, [5]Millennium Pharmaceuticals, 75 Sidney Street, Cambridge MA 02139, USA

Protein N-myristoyl transferase (NMT) catalyses the transfer of myristic acid to the N-terminal glycine of a number of proteins in eukaryotic cells. Gene knockout studies in a range of pathogenic fungal species, including *Candida albicans*, have confirmed the essential nature of NMT, which has encouraged the pharmaceutical industry to investigate this enzyme as a target site for antifungal drug discovery. To identify novel small molecule inhibitors of this target, a scintillation proximity assay for *Candida* NMT was developed, for high throughput screening of the Pfizer compound file. This screen identified the benzothiazole CP-123,467 as a moderately potent (IC50 = 1.4µM), reversible, competitive inhibitor, with a promising selectivity profile over human NMT (IC50 > 10µM). This compound was used as the lead for a medicinal chemistry program to synthesise more potent analogues, and achieve whole cell antifungal activity. Guidance for this program was provided by 3-D structural analysis of inhibitor-NMT co-crystals, in combination with traditional biochemical screening. Using this approach, a highly potent series of *Candida* NMT inhibitors were synthesised (IC50 < 20nM), which showed excellent selectivity over human NMT (IC50 >10µM), and promising mechanism-linked antifungal activity (*Candida* MIC < 5µg/ml).

1100 The HIV regulatory protein Tat and its cellular cofactors as drug targets

JONATHAN KARN
MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH

Activation of cellular genes typically involves control of transcription initiation by DNA binding regulatory proteins. The human immunodeficiency virus transactivator protein, Tat, provides the first example of the regulation of viral gene expression through control of elongation by RNA polymerase II. In the absence of Tat, initiation from the viral promoter in the long terminal repeat (LTR) is efficient but transcription is impaired because the promoter engages poorly processive polymerases that disengage from the DNA template prematurely. Activation of transcriptional elongation occurs following the recruitment of Tat to the transcription machinery via a specific interaction with an RNA regulatory element called TAR, a 59-residue RNA leader sequence that folds into a specific stem-loop structure.

After binding to TAR RNA, Tat stimulates a specific protein kinase called TAK (Tat-associated kinase). TAK is composed of two subunits, a kinase CDK9, which is structurally analogous to the CDK2 kinase required for cell cycle regulation and a novel cyclin, called cyclin T1. Cyclin T1, also participates in TAR RNA recognition. Tat is able to form a ternary complex with TAR RNA and cyclin T1 by recognising RNA sequences found the apical loop of TAR RNA.

Several of the substrates for the TAK kinase have now been identified. First, after activation of the kinase by Tat, TAK is able to hyperphosphorylate the large of subunit of the RNA polymerase II carboxyl terminal domain. Recently, we have also found that a factor involved in promoter clearance and elongation, SPT5, is hyperphosphorylated by the TAK kinase in parallel to the phosphorylation of the RNA polymerase CTD. Now that the main features of the Tat activation mechanism are understood, several attractive approaches to drug discovery present themselves. First, it is possible to inhibit Tat activation of transcription by blocking the interactions of Tat and cyclin T1 with TAR RNA. Second, it is possible to inihibit the TAK kinase directly using substrate analogues. Additional approaches, invcluding the targeting of cyclin T1 will become possible as structural information about the protein becomes available.

1145 Use of 3-D structural data to design new inhibitors to be active against drug resistant organisms

J. REN[1], L.E. BIRD[1], P. CHAMBERLAIN[1], L. DOUGLAS[1], C. NICHOLS[1,2], D.I. STUART[1,2] and D.K. STAMMERS[1,2]
[1]Structural Biology Division, The Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford OX3 7BN, [2]Oxford Centre for Molecular Sciences, New Chemistry Laboratory, South Parks Road, Oxford OX1 3QT

The development of drug resistance is an increasing problem in efforts to treat microbial infections. In the case of HIV the use of multi-drug combinations has lead to dramatic decreases in death rates from AIDS. However the rapid turnover of HIV leading to selection of escape mutants means that drug resistance is continually emerging. In the future more drugs with activity against a range of mutant HIV will be required. Drugs aimed at HIV reverse transcriptase (RT) fall into two categories: firstly, nucleoside analogue inhibitors (NRTIs), such as AZT, ddI and 3TC, and secondly the so-called non-nucleoside inhibitors (NNRTIs), which are chemically diverse compounds that binding at an allosteric site. NNRTIs are now established as part of multi-drug chemotherapy of HIV infection with nevirapine, delavirdine and efavirenz in clinical use. 'First-generation' NNRTI drugs, (e.g. nevirapine

or delavirdine) are very susceptible to the effects of single point resistance mutations within RT, whereas the more recent 'second-generation' drugs, including efavirenz, demonstrate much greater resilience to such mutations.

Our overall aim is to use structural data to develop novel anti-RT drugs that target mutant HIV resistant to current inhibitors. We have worked for many years to obtain detailed structural information on the NNRTI-RT system and have built up a database of several dozen structures. The issues we have addressed include: What is the mechanism of inhibition by the NNRTIs? What are the key features responsible for binding the chemically diverse NNRTIs within RT? Currently we are addressing further questions: What are the structural bases for mechanisms of drug resistance for different mutations? Why are the second generation NNRTIs more resilient to the presence of resistance mutations? The underlying question is of course, how can we use this structural knowledge to design better compounds? Our structures form a matrix where the parameters are, the presence or absence of NNRTI, the type of compound bound and the type of RT (wild type and drug resistant mutants). We are able to propose plausible answers to the questions raised above and there is reason to hope that new compounds can be developed to tackle HIV mutant forms resistant to current drugs.

1405 The impact of genomics on the search for novel antimicrobial agents

AILEEN ALLSOP
VP and Head of Infection Therapy Area, Astra Zeneca, Macclesfield

Over the past 40 years the search for new antibiotics has been largely restricted to well known compound classes active against a standard set of drug targets. Although many effective compounds have been discovered, insufficient chemical variability has been generated to prevent a serious escalation in clinical resistance. Recent advances in genomics have provided an opportunity to expand the range of potential drug targets and facilitated a fundamental shift from direct anti-microbial screening programmes towards rational target-based strategies. Target selection can in this way be enhanced by data comparison at the genome level, enabling the best possible choice, based on desired properties of potential agents in the clinic, such as microbiological spectrum and selectivity. Following target selection, a rational approach enables "designer" chemistry in the optimisation of interaction between compound and target at the molecular level.

No matter how well informed the choice of target, there are still many challenges remaining in the search for new antibiotics. The discovery and optimisation of compounds which fulfil clinical demands using the advantages of rational, molecular strategies rely on the ability to ensure that target interaction and antimicrobial activity are linked. It is only recently that "second generation" genomics technologies have become available to investigate the response of an entire microbial cell to challenge with a drug-candidate. In this way the application of genome-based technologies such as expression profiling and proteomics will lead to further changes in the drug discovery paradigm by combining the strengths and advantages of target-based approaches with investigation of antimicrobail activity.

Antibiotics of the future will still incur resistance, however with a variety of new mechanisms in use and more specific spectra, the hope is to create sufficient choice in the clinic to prevent reliance a small number of drug-classes.

1450 Characterisation of the mode of action of novel fungicides

D.W. HOLLOMON[1] and T. JOSEPH-HORNE[2]
[1]Dept of Agicultural Sciences, [2]Dept of Biochemistry, University of Bristol, BS 41 9AF

Fungicides control most plant diseases, but new modes of action are needed to combat resistance. Chemical leads for fungicide synthesis programmes come from natural products, targets existing in weeds and insects, technologies surrounding genomics and high throughput screening, or random screening of chemical libraries. We describe approaches used in our laboratory to identify the modes of action of new fungicide groups with established biological activity, and which are already used in practice. We emphasise how molecular biology and imaging can be integrated with biochemistry and physiology to identify the target site, and the metabolic context in which activity is expressed. Initial work explores biological activity by defining what stage in the life cycle of the target pathogen the fungicide acts, and its intrinsic activity. Resistant mutants are useful, although not always reflecting target site changes. Differential display and related techniques offer powerful ways to identify effects on gene expression. Using total RNA from resistant and sensitive powdery mildew conidia exposed the role of quinoxyfen, in the signalling pathway controlling infection. Subsequent cloning, sequencing and RT-PCR showed that quinoxyfen prevented down-regulation of a GTP-ase activating protein (GAP), keeping the corresponding ras-G protein "locked off", thereby blocking signals required to initiate key infection structures. Molecular techniques such as two-hybrid screening allow further exploration of the G-protein pathway to identify exactly how quinoxyfen interferes with it. Other approaches were needed to explore how the novel hindered silyl amide fungicide, silthiofam, acts. A series of metabolic profile experiments pointed to interference with energy production, and a combination of biochemical, spectroscopic and fluorescent probe studies revealed that silthiofam prevented ATP export from mitochondria. The ATP-ADP translocase was cloned and sequenced, but exactly how silthiofam blocks ATP export remains to be clarified. Anilinopyrimidine fungicides interfere with both secretion of enzymes required for infection, and biosynthesis of sulphur containing amino acids. Biochemical and molecular approaches alone have so far failed to identify a common target causing these effects. However, recent experiments using molecular probes suggest a rapid effect on membrane integrity, emphasising the potential of imaging techniques which allow fungicide action to be observed *in situ*.

1535 Efflux pump proteins as possible targets

O. LOMOVSKAYA
Microcide Pharmaceuticals Inc, USA

Efflux mechanisms have become broadly recognized as major components of resistance to many classes of antibiotics. Some efflux pumps selectively extrude specific antibiotics, while others referred to as multidrug resistance (MDR) pumps, expel a variety of structurally diverse compounds with differing antibacterial modes of action. Genes encoding many MDR pumps are normal constituents of bacterial chromosomes. Thus, bacteria have the potential to develop multi-drug resistance without acquisition of multiple specific resistance determinants. Genes encoding some MDR pumps are expressed constitutively in wild-type cells. As a result these cells have basal levels of efflux activity, contributing to decreased antibiotic susceptibility. This intrinsic resistance may be low enough for the bacteria still to be susceptible to therapy. However, they would be even more susceptible if efflux pumps were rendered non-functional, allowing lower doses of antibiotics to be used in therapy. This could be especially important for antibiotics with narrow therapeutic indices.

These facts taken together have led to the recognition of numerous potential beneficial consequences of the inhibition of efflux pumps in improving the clinical performance of various antibiotics, and prompted us to initiate programs to discover and develop efflux pump inhibitors.

However, while inhibition of efflux pumps appears to be an attractive approach for improving the clinical efficacy of antibiotics that are substrates of such pumps, it is important to identify antibiotics and target bacteria for which this approach would be the most productive. Several factors should be considered, such as 1) the prevalence of efflux-

mediated resistance; 2) the multiplicity of efflux pumps; 3) other mechanisms besides efflux that contribute to resistance to a particular antibiotic; and 4) interactions between different mechanisms of resistance. It will be demonstrated that the efflux pump inhibitory approach appears to be particularly attractive when applied to fluoroquinolones. Inhibition of efflux pumps is expected to (i) decrease intrinsic resistance, (ii) significantly reverse acquired resistance, and (iii) decrease the frequency of emergence of *P. aeruginosa* mutants highly resistant to fluoroquinolones.

The potential benefits of broad-spectrum efflux pump inhibitors (EPIs) prompted us to screen our synthetic compound and natural product libraries to search for such inhibitors. Secondary assays were also developed to identify leads with exquisite activity as inhibitors. Features and activity of one such lead, MC-207,110, an inhibitor of multiple MDR pumps from gram-negative bacteria, will de described in details.

POSTERS

Mi1 01 Expression and characterisation of thymydilate synthase from *Salmonella typhi*

SAMIRA BENYOUCEF, MURRAY A. SKINNER, BEN CROSSETT and KATHERINE A. BROWN
Dept of Biochemistry, Imperial college of Science, Technology and Medicine, London SW7 2AY

Thymidylate synthase (TS) is an enzyme that is crucial for DNA synthesis, both in eukaryotic and prokayotic cells. It is part of the sole *de novo* pathway for thymidine synthesis. TS catalyses the reductive methylation of dUMP by 5,10-methyltetrahydrofolate to generate thymidylate (dTMP) and dihydrofolate. Because this enzyme is required for the production of dTMP, an essential precursor of DNA biosynthesis, TS represents an important drug target.

We have initiated studies involving the isolation and characterisation of TSs from *Salmonella typhi*, the causative agent of typhoid fever in man. The genes encoding these enzymes are isolated form the strain CT18, which is a recent clinical isolate from Vietnam and which was found to be multiple drug resistant. Due to high resistance to front-line antimicrobials, third generation cephalosporins and mainly fluoroquinolones are currently used against *S. typhi*. However, resistance to third generation cephalosporins by *S. typhi* has been recently reported. This work includes expression and purification of chromosomal and plasmid TS in order to screen for specific TS inhibitors that are selective for bacterial TS with respect to human TS.

Mi1 02 Structural features needed for delivery of antimicrobial peptides by peptide transporters

SONA GUPTA, BARRY M. GRAIL, NEIL J. MARSHALL, GILLIAN M. PAYNE, MICHAEL J. UNGURS and JOHN W. PAYNE
School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

Microbial peptide transporters are able to transport virtually all small peptides (2-5 residues), in a sequence-independent manner. By using a combination of molecular modelling, and biochemical and biophysical assays with isogenic transporter mutants and purified transport proteins, we have determined the precise conformational forms of peptides, termed "molecular recognition templates" (MRTs), recognised by the three archetypal peptide transporters of *Escherichia coli*: namely, dipeptide- (Dpp), tripeptide- (Tpp) and oligopeptide permease (Opp).

Various natural antimicrobial peptides, e.g., valclavam, lindenbein and tabtoxin, rely upon peptide transporters for entry into microorganisms to effect their inhibitory activities. Modelling of the solution structures of several such compounds and a range of alternative, theoretical analogues has shown that the conformers of the natural compounds closely match those required by these transporters; some being specific for only one transporter, e.g. lindenbein, and others for several transporters e.g., valclavam. These studies show how the natural compounds have been selected to optimise delivery by peptide transporters in preference to others that match the MRTs less well and, consequently, would have poorer bioactivity. Thus, structural specificities of peptide transporters have provided a strong selective pressure on evolution of biosynthetic pathways for such antimicrobial peptides.

Mi1 03 Application of genomics and proteomics in the characterisation of *Burkholderia pseudomallei*

KAREN E. KEITH[1], BEN CROSSETT[1], PETRA C.F. OYSTON[2], RICHARD W. TITBALL[2] and KATHERINE A. BROWN[1]
[1]Dept of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, [2]DERA, CBD, Porton Down, Salisbury, Wiltshire SP4 0JQ

Burkholderia pseudomallei is the causative agent in both humans and animals of melioidosis, a disease that is endemic in South East Asia and Northern Australia. Recent improvements in awareness of melioidosis coupled with an increase in the number of laboratories isolating and identifying *B. pseudomallei* has led to an increase in the number of reported cases each year. There has not only been an increase in the number of cases within countries which have long been associated with melioidosis (Thailand, Singapore, Malaysia and Australia) but also in a number of countries now thought to be endemic such as the Philippines, China and Puerto Rica. It is not known if the increase in reported cases is linked to a genuine increase in the incidence of the disease.

Melioidosis causes one fifth of all community-acquired septicaemias in Thailand and if left untreated has a fatality rate of 95%. Treatment with antibiotics is hampered by naturally occurring resistance to a wide range of antibiotics, which includes penicillin, first and second generation cephalosporins and many aminoglycosides. The sequencing of the genome is currently under way at the Sanger centre and will provide a wealth of information on the number and type of antibiotic resistance genes present in *B. pseudomallei*. We have initiated studies involving the analysis and isolation of β-lactamases, including the identification of a class D β-lactamase in *B. pseudomallei.* The genomic sequence will facilitate the identification of proteins during initial work on the construction of the proteome map of *B. pseudomallei.*

Mi1 04 The interactions of the *Yersinia pestis* virulence proteins LcrV and LcrG

DANIEL G. LAWTON[1], JIM HILL[2], RICHARD W. TITBALL[2] and KATHERINE A. BROWN[1]
[1]Dept of Biochemistry, Imperial College, Exhibition Road, London SW7 2AY, [2] Biomedical Sciences Dept, DERA Porton Down, Salisbury, Wiltshire SP4 0JQ

LcrV and LcrG are key proteins in the type III secretion system of *Yersinia*. LcrV is a secreted protein, required for the translocation of Yop effectors into the mammalian cell. It is also regulatory, and enhances Yop expression and secretion under inducing conditions. This regulation is mediated through a competitive interaction with LcrG. LcrG is believed to be an intracellular "gate", blocking type III secretion under non-inductive conditions. The multimerisation of LcrV and its interaction with LcrG may be facilitated by coiled-coil domains. These are important for several protein interactions in type III secretion systems. Indeed predicted coiled coil domains are present in both LcrV and LcrG.

The aim of this work is to probe the importance of such domains in the interaction of LcrV both with itself and with LcrG. In this study LcrV, LcrG and site directed mutants in coiled-coil domains have been cloned, expressed and purified to homogeneity. These wild type and mutant proteins have been studied by ELISA and SPR techniques. Furthermore, complementation of *Yersinia pseudotuberculosis* strains was used to address the significance of these mutants.

Mi1 05 Tailoring the conformations of a synthetic inhibitor of microbial cell wall biosynthesis for optimal activity

NEIL J. MARSHALL, SONA GUPTA and JOHN W. PAYNE
School of Biological Sciences, University of Wales Bangor, Bangor, Gwynedd LL57 2UW

Cell wall biosynthetic enzymes, such as glucosamine-6-phosphate synthetase, involved in the synthesis of N-acetyl glucosamine, are attractive targets for the design of antimicrobial agents. However, although the enzyme is inhibited *in vitro* by various glutamine analogues, activity against whole cells is frequently negligible because the inhibitors fail to gain access to the cytoplasmic location of the enzyme.

The glutamine mimetic N^3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid (FMDP) shows activity against purified enzyme *in vitro* but is only active against whole cells when incorporated into a peptide. For several such peptide analogues we assayed their antimicrobial activities, their binding to purified peptide binding protein and determined their conformations in solution using molecular modelling. Positive correlations were found between antimicrobial activities and inhibitor binding. Molecular modelling showed that the most inhibitory compounds had conformational profiles that best matched the molecular recognition templates (MRTs) of peptide transporters, whereas less active analogues had poorly matching conformer profiles. Thus, molecular modelling of such antimicrobial peptides can be used as a predictor of their biological activities *in silico* permitting rational design and synthesis at an early stage in a drug development programme.

Mi1 06 Molecular characterisation of the Reg proteins of *Rhodobacter sphaeroides*

C.A. POTTER[1], M.P. WILLIAMSON[2], P.J.F. HENDERSON[1], S.E.V. PHILLIPS[1] and M.K. PHILLIPS-JONES[1]
[1]School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2-9JT, [2]Dept of Molecular Biology and Biotechnology, University of Sheffield, S10 2TN

In the photosynthetic Gram negative bacterium *Rhodobacter sphaeroides* a two-component signal transduction pathway is involved in sensing changes in external environmental aerobiosis/anaerobiosis conditions and exerts global regulatory control over many metabolic processes including photosynthesis, reductive assimilation of carbon dioxide and nitrogen fixation. It comprises a histidine protein kinase (RegB), which is involved in sensing external oxygen concentrations and a two-domain transcriptional activator protein (RegA), which functions as the response regulator. Our aim is to characterise the interaction of these proteins at the molecular level in order to understand the mechanism of signal transduction, how differential global regulatory control is achieved, and use this model system to gain insights into the exploitation of two-component systems as potential targets for novel antibiotic therapies.

We have transferred the *RegA* gene to plasmid pET14b and overexpressed the RegA protein in *Escherichia coli*. The resulting $RGSH_6$ -RegA fusion protein has been purified and partially characterised. RegA domain fragments have also been overexpressed and purified so that the interactions of the two domains can be elucidated at the molecular level.

Mi1 07 Enzyme-mediated capsule degradation as a novel approach to the treatment of bacterial meningitis

MARIA B. REDPATH[1], DANIEL R. LEGGATE[1,2], J. PAUL LUZIO[2] and PETER W. TAYLOR[1]
[1]Dept of Pharmaceutics, School of Pharmacy, Brunswick Square, London WC1N 1AX, [2]Wellcome Trust Centre for Molecular Mechanisms in Disease, University of Cambridge, Hills Road, Cambridge CB2 2XY

Escherichia coli strains expressing the K1 capsular polysaccharide are responsible for a high proportion of cases of bacterial neonatal meningitis, a life-threatening disease affecting infants in the first month of life. The capsule is a linear homopolymer of α-2,8-linked *N*-acetylneuraminic acid and, due to its structural relatedness to polysialic acids expressed in developing mammalian tissues, protects the bacterial cell from immune surveillance. Removal of the capsule *in vivo* is likely to sensitise neuroinvasive bacteria to humoral and cellular components of the immune system and facilitate their removal by these processes. In order to examine this hypothesis, we have identified an enzyme associated with the tail spike assemblage of K1-specific bacteriophages which hydrolyses α-2,8-linkages within the polymer and we are examining the capacity of endosialidase, when administered parenterally, to effect removal of K1 strains from tissues of experimentally infected animals. We have cloned the gene encoding the K1-specific endosialidase and expressed recombinant fusion proteins in *E. coli* BL21(DE3). The recombinant protein, containing a histidine tag at the N-terminus, has been purified by immobilised metal affinity chromatography. A process has been developed to produce high yields of highly purified, recombinant endosialidase. Recombinant enzyme is under investigation with regard to its capacity to prevent and cure systemic *E. coli* K1 infections in neonatal rats.

Mi1 08 Identification of genes involved in two-component signal transduction in *Burkholderia pseudomallei*

SUTHA SANGIAMBUT[1], MAGDY MAFFOUZ[2], MARTYN L. GILPIN[2], KATHERINE A. BROWN[1] and NEIL FAIRWEATHER[1]
[1]Dept of Biochemistry, Imperial College, Exhibition Road, London SW7 2AY, [2]Dept of Biological Sciences, University of Plymouth, Plymouth, Devon PL4 8AA

Burkholderia pseudomallei is the causative agent of melioidosis, a disease that is a major cause of morbidity and mortility in Southeast Asia and Northern Australia. This organism is a facultative intracellular pathogen capable of remaining dormant in host macrophages for many years and the latency period can be activated to develop into an acute, fulminating and fatal infection when an individual becomes immunocompromised. *B. pseudomallei* is resistant to a large number of antibiotics and future therapeutic intervention could involve the development of a vaccine.

In this context, the aim of the project is therefore to identify and characterise appropriate genes and gene products, which may be suitable for generating mutants that could be used as live vaccines. Using degenerate oligonucleotide primers and *B. pseudomallei* genomic DNA, we have identified by PCR a DNA fragment which encodes part of an ORF that shows 66 % amino acid similarity to *Salmonella typhimurium* PhoP, a response regulator gene involved in two-component signal transduction. It has been previously shown that *phoP* mutations in *Salmonella typhimurium* and *Salmonella typhi* are attenuated and may be potentially useful as live vaccines. This PCR fragment has been used to screen a *B. pseudomallei* genomic library in phage λ to identify the entire ORF of the *B. pseudomallei* response regulator gene.

Mi1 09 Expression and purification of EPSP synthases from pathogenic organisms

JANE E. SAUNDERS, MURRAY A. SKINNER, PILANEE VAITHANOMSAT and KATHERINE A. BROWN
Dept of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY

The increase and spread of multi-drug resistant strains of pathogenic bacteria has led to a growing need for new antimicrobial agents. The shikimate pathway, which leads to the biosynthesis of ring-containing compounds, is an important potential drug target. This pathway is present in bacteria, microbial eukaryotes, plants and apicomplexan

parasites, but absent in mammals. The *aroA*-encoded 5-*enol*pyruvylshikimate-3-phosphate (EPSP) synthase, the primary target for the broad-spectrum herbicide glyphosate, catalyses the sixth step of the pathway, converting shikimate-3-phosphate and phosphoenolpyruvate to EPSP and phosphate.

The aim of this study is to express and purify EPSP synthases from *Pseudomonas aeruginosa* and *Francisella tularensis* as a prerequisite to future structure-function studies. The *P. aeruginosa aroA* gene has been subcloned into the expression vector pTrc99A, overexpressed and purified by a three-step purification. The *F. tularensis aroA* open reading frame has been successfully amplified and subcloned into a number of expression vectors. Expression and purification of the resulting EPSP synthase has been completed.

Mi1 10 Dehydroquinate synthase – inhibitor complexes

MITHILA SHAFIQ[1], MURRAY A. SKINNER[1], ELISABETH P. CARPENTER[1], ALASTAIR R. HAWKINS[2], JOHN FROST[3] and KATHERINE A. BROWN[1]
[1]Dept of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, [2]Dept of Biochemistry and Genetics, University of Newcastle upon Tyne, NE2 4HH, [3]Dept of Chemistry, Michigan State University, East Lansing, Michigan 48824-1322, USA

Antibiotic resistance has presented a serious challenge in the treatment of infectious diseases in the last decade. As the prevalence of multidrug-resistant strains of bacterial pathogens increases, there is an increasing need for identifying new antimicrobials and antimicrobial targets. Dehydroquinate synthase (DHQS), is an NAD^+-dependant metalloenzyme. It is the product of the gene *aroB*, which is part of the shikimate pathway of enzymes involved in the biosynthesis of aromatic amino acids and other ring containing compounds including folate and para-aminobenzoate. This pathway is present in microorganisms, plants and certain parasites but is absent in humans. The products of this pathway are essential to the survival of microbial pathogens. Mutation of the *aroB* gene in *Salmonella typhimurium*, has been shown to result in a strain with attenuated virulence in mice. Taken together, this suggests that chemical inhibition of DHQS from microbes could have bacteriostatic or even bacteriocidal effects.

Recombinant DHQS from several microorganisms has been over-expressed and purified. Steady state kinetic parameters have also been obtained for these enzymes. Inhibitory studies of DHQS have been initiated. These and complementary structural studies will be presented here.

Mi1 11 Investigation of a herbicide target: evolution of a pathway and of enzyme function

PAPINYA SIRIMONGKOLKASEM, MURRAY A. SKINNER, KATHERINE A. BROWN and JEREMY O. BAUM
Dept of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY

The biosynthesis of the branched-chain amino acids valine and isoleucine involves four steps from pyruvate to the amino acid. This pathway occurs in many lower organisms, but not in higher eukaryotes such as man. The first of these steps involves the enzyme acetohydroxyacid synthase (AHAS), which is also the key regulated enzyme in the pathway. It is the target of a number of herbicides, and is also a potential target for other applications such as treatment of tuberculosis. It is highly homologous to the enzyme pyruvate oxidase, which has a similar but distinct catalytic activity. Although they are both flavoenzymes, the FAD is only utilised in pyruvate oxidase. Using the experimental structure of pyruvate oxidase we have modelled AHAS and propose reasons for the differences in catalytic function. Such improvements in our understanding of enzyme function will help to improve the design of new ligands.

Although higher animals cannot synthesis branched-chain amino acids, we have found homologues in higher animals for the first and last steps in the pathway. The presence of AHAS in humans was unexpected. Using sequence and structural analysis we have investigated the likely catalytic capability of the human enzyme. Further work will attempt to reveal the role of this enzyme in humans. The last step is known to be involved in valine catabolism in humans. Moreover this enzyme (transaminase) has been implicated in a number of processes wholly unrelated to amino acid biosynthesis and to bacterial life. We have investigated these to try to explore how the transaminase may have evolved these new functions.

Mi1 12 Identification of *Francisella tularensis* virulence factors as a prelude to rationally designed tularemia therapeutics

MURRAY A. SKINNER[1], KEVIN W. PAGE[2], KATHERINE A. BROWN[1] and RICHARD W. TITBALL[2]
[1]Dept of Biochemistry, Imperial College of Science, Technology and Medicine, London SW7 2AY, [2]DERA, CBD Porton Down, Salisbury, Wiltshire SP4 0JQ

Francisella tularensis can cause a severe pneumonia-like infection in man. An attenuated mutant (Live Vaccine Strain, LVS) has reduced virulence in humans and is currently in use as an experimental vaccine for laboratory workers, however, its efficacy is in question. The molecular basis of attenuation of pathogen virulence in LVS is unknown and it is the molecular basis for the loss of virulence in LVS and by implication, putative virulence factors in wild-type *F. tularensis*, that are considered here. Using Representational Difference Analysis (RDA) we have identified a region of DNA within the genome of the fully virulent *F. tularensis* strain Schu4 which is absent in the LVS. By database comparison we find a deletion occurs within the open reading frame (ORF) of the *ilvB* gene, which encodes acetohydroxyacid synthase (AHAS). The open reading frames of *ilvB* from the Schu4 and LVS strains have been cloned. We note that the intact ORF from the Schu4 strain will complement an *Escherchia coli ilv*$^-$ auxotroph, whilst the *ilvB* gene from LVS will not. Based upon existing structural evidence for *Lactobacillus plantarum* pyruvate oxidase, an AHAS homologue, we have built a molecular model of *F. tularensis* AHAS and have identified potential catalytic residues. We have tested the importance of residues by constructing site-directed mutants in the Schu4 *ilvB* gene. The relevance of the LVS deletion with respect to the contribution to attenuation of pathogen virulence *in vivo* will be discussed. We also consider inhibition of AHAS as a novel target for anti-microbial development.

Mi1 13 The role of salmonella pathogenicity island 1 & 2 encoded type III secretion systems in pathogenesis of fowl typhoid

PAUL WIGLEY*, MICHAEL JONES and PAUL BARROW
Institute for Animal Health, Compton Laboratory, Newbury, Berks RG20 7NN - *Current Address: School of Pharmacy & Pharmaceutical Sciences, De Montfort University, The Gateway, Leicester

Fowl typhoid (FT) is a severe systemic disease of both young and adult chickens, characterised by mortality rates of up to 90%. It is of considerable economic importance in countries where intensive rearing of poultry is still in its infancy. FT is caused by *Salmonella enterica* serovar Gallinarum (*Salmonella gallinarum*) which rarely causes disease in species other than chickens and related birds. Host specificity is expressed at the level of the reticuloendothelial system, but other than the requirement of a 85kb virulence plasmid little is known regarding the mechanisms of pathogenesis of *S. gallinarum*.

In this study mutations were made in the *Salmonella* pathogenicity island 1 (SPI 1) (*spa*S$^-$) and SPI 2 (*ssaU*$^-$) type III secretion systems (TTSS). The mutants were tested *in vitro* for invasion into non-phagocytic chicken cells, and for their ability to persist within the chicken macrophage-like cell line (HD11). As in other salmonellae, mutation in SPI 1 but not SPI 2 reduced the invasiveness of the bacterium significantly

when compared with the parent strain. In contrast SPI 2 mutants failed to persist longer than 4 hours in chicken macrophages, whilst both the parent strain and SPI 1 mutant both persisted for at least 24 hours. Virulence was tested by orally infecting 3-week old Rhode Island Red chickens with 10^8 cfu of the mutants and parent strain. A mortality rate of 60% (6/10) was seen with both the parent and SPI 1 mutant strains. No mortality was seen in the SPI 2 mutant infected birds. By measuring organ counts it was found that the SPI 2 mutant was not taken up to the spleen or liver and failed to cause any pathology. In contrast the SPI 1 mutant and parent strain multiplied in the spleen and liver causing pathology consistent with fowl typhoid. Following intravenous infection with 10^4 cfu of both mutants and parent strain, bacteria were taken up by the spleen within a few hours. However the SPI 2 mutants were cleared within 24 hours, whereas the SPI 1 mutant multiplied in the spleen and liver causing disease. These findings indicate that, in common with *S. typhimurium* infection in the mouse, *S. gallinarum* requires the SPI 2 but not the SPI 1 TTSS to cause systemic typhoid-like disease.

Mi1 14 Control of biofilm formation by photoactivation of surface bound phenothiaziniums

A.A. MATCHETT, M. WAINWRIGHT and S.L. PERCIVAL
Pathogen and Biofilm Research Group, Dept of Biological Sciences, University of Central Lancashire, Preston, Lancashire PR1 2HE

In light of ever increasing evidence of antibiotic resistance in pathogens, especially when situated within a biofilm, alternative therapies must be considered. These new 'microbially unassailable' therapies could alleviate, possibly even replace, our ever-increasing reliance on stronger more potent antibiotic cocktails, with some.

Several previous investigations have established that bacteria, and bacteria within biofilms, are sensitive to the photosensitzing action of a variety of light absorbing dyes. The application of these light activated molecules to the killing of bacteria has been putatively described as Photodynamic Antimicrobial Therapy (PACT). The localised ability of the light activated molecule to instigate redox reactions and/or form highly reactive singlet oxygen is *modus operandi* of these particular antimicrobials.

The effectiveness of organic polymer bound cationic azines as surface photosensitizers is being investigated by the formation of gram-positive and gram-negative biofilms on an artificial multiport sampling catheter *in vitro*. The sample surfaces are subsequently treated with red light and examined by a combination of techniques (including viable cell counts, epiflourescence, and scanning electron microscopy).

Mi1 15 Comparison of the inherent susceptibility of medically device-related materials to biofilm development

A.A. MATCHETT and S.L. PERCIVAL
Pathogen and Biofilm Research Group, Dept. of Biological Sciences, University of Central Lancashire, Preston, Lancashire PR1 2HE

With the use of implantable devices increasing with an ageing population and other high-risk groups, and the rising incidence of antibiotic resistance in infection, medical hygiene has come under greater scrutiny. Conventional prophylaxis does not entirely reduce the risk of infection in the case of implantable devices, hence the requirement of additional antimicrobial measures. A wide variety of polymer modifications have been published, for example, to reduce surface adhesion, or with surface bound antimicrobials incorporated.

This has prompted an investigation into the susceptibility, or resistance of these materials *per-se*, to bacterial contamination and colonization. Specifically modelling the development of biofilms that may lead to infection, the study investigates various materials including several polymers used in catheters with particular interest in the surface interaction with the biofilm.

An *in vitro* artificial multiport sampling catheter model connected to a chemostat is used to determine the susceptibility of the materials to gram-positive and gram-negative bacterial

contamination, colonization and initial biofilm formation. The sample surfaces are examined by standard techniques including viable cell counts, epiflourescence, and scanning electron microscopy.

Mi1 16 Identification of an auxotrophic mutant, that is protective in the mouse model of *B. pseudomallei* infection

T. ATKINS[1], K. MACK[1], R. PRIOR[1], P. RUSSELL[1], P.C.F. OYSTON[1], R.W. TITBALL[1] and G. DOUGAN[2]
[1]CBD, Porton Down, Salisbury, Wiltshire SP4 OJQ,
[2]Imperial College of Science, Technology and Medicine, London

Burkholderia pseudomallei occurs in tropical and subtropical climates and is the causative agent of melioidosis. Melioidosis used to be considered a relatively rare disease, but with improved diagnostic techniques and an increase in global travel, melioidosis is becoming more common and being isolated from more varied environments.

We have utilised standard transposon mutagenesis in combination with screening on defined growth media to identify auxotrophic mutants of *B. pseudomallei*. Animal infection studies were used to identify mutants, which were attenuated for infection of a mouse model. One of these bacterial mutants was found to have the transposon inserted into the *ilv* I gene, which encodes the catalytic sub-unit of the acetolactate synthase enzyme. This mutant was shown to be auxotrophic for the amino acids leucine, valine and isoleucine. A protective effect was seen in mice vaccinated with the *ilv* I^- mutant and subsequently challenged with wild-type *B. pseudomallei*.

Mi1 17 Effect of the *Mycobacterium bovis* RD13 deletion in the expression of *tap* gene, which encodes an efflux pump present in *Mycobacterium tuberculosis* complex

P.E.A. SILVA, V. MICK, C. MARTIN and J.A. AÍNSA
Departamento de Microbiología, Universidad de Zaragoza, calle Domingo Miral s/n, 50009Zaragoza-Spain - Email: pedre@posta.unizar.es

The sequencing of the genome of *M. tuberculosis* has revealed at least 22 putative genes encoding drug transporters. The *tap* gene (Rv1258c) encodes an efflux pump conferring resistance to aminoglycosides and tetracycline. Downstream of tap gene and in the same transcriptional direction (possibly forming an operon) there are three other genes: Rv1257c encoding a putative oxireductase, Rv1256c encoding a putative cytochrome P450, and Rv1255c encoding a putative transcription regulator similar to AcrR and TetR regulators. We have identified one deletion (RD 13) in *M. bovis, M. africanum y M. microti* affecting these three genes. Considering that *tap* gene could be co-regulated with Rv1255c, Rv1256c and Rv1257c, one interesting hypothesis suggests that the absence of the putative regulator Rv1255c in *M. bovis* should produce differences in the expression levels of the tap gene in comparison with M. tuberculosis. Another question to address is whether the expression of the tap gene is growth-phase regulated as it happens to other efflux pumps. We have constructed a plasmid carrying a traductional fusion of the tap gene with its possible promotor region and the *lacZ* reporter gene. In *M. tuberculosis* and *M. bovis* carrying this construction the β-galactosidase activity was growth-phase dependent and the highest levels of activity were observed during the exponential phase of growth. When comparing β-galactosidase activity from *M. bovis* and *M. tuberculosis* transformants, we observed highest levels of expression in *M. bovis*. In summary, our results

indicate that *tap* gene is growth-phase regulated, and the presence of the deletion RD 13 in *M. bovis*, which removes the putative regulator encoded by Rv1255c, possibly explains the higher levels of expression of *tap* gene observed in *M. tuberculosis*.

MICROBIAL INFECTION GROUP
Activities and actions of antimicrobial peptides

TUESDAY 27 MARCH 2001

0930 Activities and actions of antimicrobial peptides from higher eukaryotes

TOMAS GANZ
Professor of Medicine and Pathology, University of California, Los Angeles CHS 37-055, 10833 Le Conte Ave., Los Angeles, CA 90095-1690, USA

Antimicrobial peptides are small (fewer than 100 amino acids) polypeptides that our found in host defense contexts in animals and plants. Nearly all are cationic and amphipatic, a feature that facilitates their interaction with microbial membranes. Whereas most of the peptide antibiotics derived from microbes are the products of complex metabolic pathways and contain modified amino acids, the antimicrobial peptides of animals and plants are encoded by genes, produced on ribosomes, and contain few or no modified amino acids. Antimicrobial peptides present a bewildering variety of structural species ranging from simple amphipatic alpha-helices to multiply disulfide-linked beta-sheet rich forms. The mechanism of action of antimicrobial peptides involves the permeabilization of microbial cell membranes by the formation of pores or other disruptive structures but additional intracellular targets for peptides may also be important. Experiments with artificial membranes suggest that the relative selectivity of antimicrobial peptides for microbial as opposed to host membranes is due to differences in membrane composition: microbial membrane lipids are more anionic, favoring the entry of the peptides. In mammals, the predominant gene families are the defensins and the cathelicidins, both of which are found in leukocytes and epithelia. The defensins are three-disulfide peptides, 29-47 amino acids in their active form, and consisting mostly of beta-sheet. They are found in the azurophil granules of neutrophils, a granule type which is destined to fuse to phagocytic vacuoles. Other defensins are also stored in the granules of intestinal Paneth cells and produced by various epithelia, either constitutively, or in response to infections. They are broadly microbicidal at microgram/ml concentrations and are variably and competitively inhibited by increasing salt and serum concentrations. In contrast, cathelicidins are present in the specific granules of neutrophils, granules that are destined for secretion into extracellular space. They are also expressed in the testis and inflamed epithelia. Cathelicidins vary greatly in structure and the conserved part is a 100 amino acid propeptide which is most often proteolytically removed during or after secretion. Lower vertebrates and invertebrates have an even more diverse repertoire of antimicrobial peptides. There is increasing evidence that antimicrobial peptides are important effectors of innate immunity. Moreover, their broad spectrum of antimicrobial activity has attracted the attention of pharmaceutical companies and several antimicrobial peptides have been developed as topical antimicrobials.

1015 Genomic organisation and function of members of the murine and human beta defensin gene family

GILLIAN MORRISON, FIONA KILANOWSKI, DONALD DAVIDSON, MARK ROLFE and JULIA DORIN
MRC Human Genetics Unit, Western General Hospital, Edinburgh EH4 2XU

Studies on the function of beta defensins have mainly been carried out in *in vitro* systems and in an effort to overcome the problems associated with these systems it is one of our aims to use the mouse as a model to study beta defensin function and dysfunction *in vivo*.

Four murine beta defensins have been identified and reduced stringency hybridisation experiments show evidence for the existence of further members of this gene family. We have previously shown that the alpha defensin genes (the cryptidins) and the beta defensin gene Defb1 lie within the same genomic BAC fragment on mouse chromosome 8. In order to fully characterise the murine defensin gene family we have assembled a contig which covers approximately 300kb and contains all of the known genes. We have established the genomic organisation of the previously identified beta and alpha defensins and have also identified novel beta defensin gene sequences. One genomic derived putative gene sequence was subjected to 5' RACE from mouse tracheal RNA and identified a full-length transcript which corresponds to this sequence and which we have named *Defb5*. We are currently investigating the function of the peptide encoded by this novel gene as part of our ongoing studies into the antimicrobial action of beta defensins and we are also analysing the role of these genes by creating knockout mice. We have created a *Defb1* knockout mouse but to date the mutant mice have not revealed any significant difference in their ability to cope with microbial infections in the airways. Our analysis of the expression of the other murine beta defensins in the airways of these *Defb1* knockout mice in addition to our analysis of the antimicrobial nature of synthetic murine beta defensin peptides strongly suggest the existence of functional redundancy within this gene family.

We have investigated the action of Defb1 and Defb2 synthetic peptides in addition to that of the human beta defensins HBD-1 and HBD-2 which has revealed different specificities of action between the peptides. We are also working towards a full characterisation of the human beta defensin gene family and our analysis of the genomic region which contains the known human beta defensin genes has revealed several putative coding sequences for novel beta defensins. The continued study of the antimicrobial action of the existing human and mouse beta defensin peptides in addition to the novel sequences presently being discovered will reveal additional functional differences which will have major implications towards the elucidation of gene function and the potential use of beta defensins in a therapeutic context. (*This is supported by the CF Trust and the Medical Research Council.)*

1115 Antimicrobial peptides in cystic fibrosis

MALCOLM L. WATSON, ROS M. BAIRD, JODY KIRKAM-BROWN and ANTHONY W. SMITH
Dept of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY

Cystic fibrosis (CF) lung disease is characterised by chronic infection with *Pseudomonas aeruginosa* and the concomitant inflammation and tissue damage results in a progressive decline in lung function and ultimately to patient mortality. Although relatively infrequent, infection with *Burkholderia cepacia* also occurs. CF is a result of mutation in the cAMP regulated chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), resulting in defective expression and/or function in epithelial cells. A central

question in CF is how defective CFTR results in chronic and non-resolving lung infections, despite a vigorous host response.

The airway surface liquid overlying the epithelium contains numerous antimicrobial agents, including lysozyme, lactoferrin and defensins. It has been postulated that defective CFTR results in elevated ionic strength of the airway surface liquid[1]. Although this finding is controversial (see [2]), such a change would reduce the antimicrobial activity of the airway surface liquid and hence increase survival of bacteria. Our studies[3] indicate defensin-like activity from cultured human bronchial epithelial cells that decreased growth of mucoid *P. aeruginosa*, whilst growth of *B. cepacia* isolates were not inhibited.

Although characterised as epithelial-derived antimicrobial peptides, ß-defensins have been shown to interact with the CCR6 chemokine receptor[4]. This indicates a dual role for defensins in host defence, acting both as direct antimicrobials and as amplifiers of the adaptive immune system by recruiting and activating immune cells. Such interactions are currently under investigation in our laboratories.

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1200 Role of low molecular weight protease inhibitors (secretory leukocyte protease inhibitor/elafin) in innate immunity

JEAN-MICHEL SALLENAVE
Rayne Laboratory, Centre for Inflammation Research, Edinburgh University

As part of the immediate defence against bacterial pathogens, the lung secretes antimicrobial peptides such as secretory leucocyte protease inhibitor (SLPI), elafin/elastase-specific inhibitor, lysozyme and lactoferrin. SLPI and elastase-specific inhibitor/elafin are two low-molecular weight elastase inhibitors that are mainly synthesized locally at mucosal sites. It is thought that their physicochemical properties allow them to efficiently target enzymes, such as neutrophil elastase, when released into the interstitium. They are up-regulated by "alarm signals" such as bacterial lipopolysaccharides, cytokines such as interleukin-1 and tumor necrosis factor (Am J Respir Cell Mol Biol 11, 733-741, 1994) as well as neutrophil elastase (FEBS Letters 457, 33-37, 1999). In addition, we and others have shown that these inhibitors have anti-bacterial actions against Gram-positive and Gram-negative bacteria (FEBS Letters 452, 309-313, 1999) and could therefore be considered as "defensin-like"peptides produced at sites of mucosal inflammation. We are currently using a dual strategy to overexpress the elafin gene : (i) human elafin (h-elafin) transgenic mice lines were established where h-elafin gene is expressed under the control of the mouse cytomegalovirus promoter (MCMV). Using Northern Blot and RT-PCR analysis, we have shown that all organs, including the lung express h-elafin mRNA. Ex-vivo and in vivo data show that these mice are hyporesponsive to bacterial lipopolysaccharide. (ii) Using adenovirus as a gene-transfer vector (Gene Therapy, 5, 352-360, 1998), we have recently shown that adenovirus-elafin can protect:
In vitro: alveolar epithelial cells against human neutrophil elastase or activated neutrophils-induced damage.
In vivo: C57/Bl6 mice against *Pseudomonas aeruginosa*-induced lung injury (Simpson et al, submitted).
Conclusions: SLPI and elafin/SKALP are gradually being recognized as potent locally produced elastase inhibitors whose characteristics allow them to be present at the onset of bacterial inflammation. We showed that overexpression strategies (using transgenics and adenovirus-vectors) are useful to study further their function in mice models of bacterial infections.

1225 Upregulation of tap expression in bovine skin following bacterial infection

GERRY McLACHLAN[1], PILAR ALBERDI[2] and NICK AMBROSE[2]
[1]Dept of Veterinary Clinical Studies, [2]Dept of Tropical Animal Health, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush Veterinary Centre, Midlothian EH25 9RG

Bovine tracheal antimicrobial peptide (TAP) is known to be expressed in airway columnar epithelium. In this study we demonstrate its expression in bovine skin following experimental and natural infection with *Dermatophilus congolensis*, a branching, filamentous actinomycete that infects the epidermis of ruminants. Lesions are characterised by crust formation, serous exudate and neutrophil infiltration. Experimental infections resolve without treatment in 2-3 weeks after a first infection and 1-2 weeks following a second infection. Natural chronic infections are associated with the immunosuppressive effects of infestations with the tick *Amblyomma variegatum*. We sought to examine the timing of TAP expression in our experimental system. Total RNA from skin biopsies of experimental and natural lesions and uninfected skin were analysed by RT-PCR for expression of the TAP gene. TAP expression was not detected in control skin but was upregulated on days 2,5,8,13 and 21 post first infection, with highest levels at day 8. Following the second infection TAP expression was upregulated on days 2, 5 and 8, with maximum levels on day 5. TAP expression was also upregulated in natural chronic lesions compared to control skin. A parallel study showed that the ovine gene sBD1, that has high identity to TAP, was not clearly upregulated in sheep skin following *D. congolensis* infection. TAP expression in cultured airway epithelium is rapidly upregulated via NFκB binding in response to inflammatory and infectious stimuli such as TNFα, IL-1β, LPS, LTA and muramyl dipeptide. Our study shows delayed and extended upregulation of TAP expression *in vivo* in the skin. Further studies could use this system to address mechanisms of TAP gene regulation in the stratified squamous epithelium of the epidermis, the antimicrobial activity of TAP against *D. congolensis* and possible cell chemoattractant functions.

1400 Resistance mechanisms to antimicrobial peptides in Gram-negative bacteria

EDUARDO A. GROISMAN and ARDEN ASPEDON
Howard Hughes Medical Institute, Dept Of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Ave., Campus Box 8230, St. Louis, Missouri 63110, USA

The production of antimicrobial peptides is a ubiquitous host defense mechanism utilized by vertebrates and invertebrates animal species, plants and even microbes. However, pathogenic microorganisms have evolved mechanisms that allow them to resist killing by antimicrobial peptides. In the Gram-negative pathogen *Salmonella enterica*, the PhoP/PhoQ two-component regulatory system controls resistance to the mammalian defensins, the frog-derived magainin, the insect cecropin, as well as other antimicrobial peptides. For example, when the PhoP/PhoQ system is induced during growth in low magnesium, *Salmonella* is >3,000-fold more resistance to magainin than when the organism is grown under repressing concentrations of magnesium. However, different PhoP-regulated determinants mediate *Salmonella* resistance to different antimicrobial peptides. In *Pseudomonas aeruginosa*, the main colonizer of the lung of cystic fibrosis patients, a regulatory locus termed *saf* controls resistance to antimicrobial compounds present in airway surface fluid. The *saf* locus also controls the production of secondary metabolites implicated in virulence. This suggests that *saf* in *Pseudomonas*, like *phoP* in *Salmonella* may contribute to the virulence in these two Gram-negative species by protecting the microorganism from the killing effects of host defense peptides.

1445 Antimicrobial peptides in the human mouth

D.A. DEVINE, A.S.HIGH and W. BONASS
Leeds Dental Institute, University of Leeds, Clarendon Way, Leeds LS2 9LU

The human mouth contains widely varying sites that support large and complex communities of bacteria. Frequency of infection does not reflect this microbial load and, in general, these populations are tolerated. The mechanisms determining or controlling this host-microbe balance are not fully understood, but antimicrobial peptides are likely to be important. We recently found, using RT-PCR, *in situ* hybridisation and immunohistochemistry, that normal and inflamed major and minor salivary glands produce β-defensins 1 and 2 (HBD1 & 2) and novel wound healing peptides (trefoil factors, which, in the gut are important in healing and protection, especially with respect to ulceration). Production of HBD 1, HBD 2 and the cathelicidin LL37 has also been demonstrated using oral epithelial cells. A number of studies have now shown HBD 2 and LL37 expression is inducible in all these tissues by bacteria, and commensal species appeared to be less likely than pathogenic species to elicit this response. Antimicrobial peptides also rapidly bound and killed periodontopathogenic bacteria, despite production of large amounts of extracellular proteases. It is probable that antimicrobial peptides are key components regulating commensal oral populations, modulating the pathogenicity of some species and protecting mucosal surfaces alongside molecules such as trefoil factors. Both may be significant clinically as markers of disease and as therapeutic agents.

1510 Adrenomedullin expression in pathogen-challenged oral epithelial cells

S. KAPAS and R.P. ALLAKER
Barts & the London School of Medicine & Dentistry, Turner Street, London E1 2AD

Epithelium provides the first line of defence against invading microorganisms in animals and plants. Several antimicrobial peptides have now been isolated and shown to be expressed by surface epithelial cells, such as ß-defensin, and these discoveries have led to an emerging concept that these molecules contribute to the protective barrier of the epithelium against pathogenic microorganisms. Adrenomedullin, a multifunctional peptide, is expressed by many surface epithelial cells. We have previously demonstrated that adrenomedullin has antimicrobial activity against members of the human skin, oral, respiratory tract and gut microflora. The oral cavity contains an epithelium that is permanently colonised by a microflora which includes bacteria, fungi and viruses. The oral mucosa is often subjected to trauma, yet infections in a host are rare. Oral keratinocytes were exposed to culture supernatants of four microorganisms commonly isolated from the oral cavity, *Porphyromonas gingivalis*, *Streptococcus mutans*, *Candida albicans* and *Eikenella corrodens*. It was observed that adrenomedullin was secreted from these cells and that gene expression was upregulated in the presence of the bacteria, but not with the yeast, *Candida albicans*. All microorganisms, however, induced expression of human ß-defensin 2. We propose there is a potential role for microbial products in enhancing mucosal defence mechanisms and that adrenomedullin participates in the prevention of local infection, thus contributing to host defence mechanisms.

1600 Therapeutic potential of antimicrobial peptides

R.E.W. HANCOCK
Dept of Microbiology & Immunology, University of British Columbia, 300 - 6174 University Boulevard, Vancouver, B.C. V6T 1Z3 Canada

Antibiotics are perhaps the most successful pharmaceutical. However, with the increasing development of antibiotic resistance amongst key pathogens, there is an urgent need to discover novel classes of antibiotics. We are interested in antibacterial cationic peptides which are produced by virtually all organisms, ranging from plants and insects to humans, as a major part of their immediately-effective, non-specific, innate immune defence against infections. Since natural sources of these peptides do not yield sufficient quantities to permit them to be utilized therapeutically, and since peptide synthesis is expensive, we developed a recombinant DNA procedure for making such antimicrobial peptides in bacteria. Biochemical and animal model studies demonstrated that these peptides had potential as stand-alone, broad-spectrum antibiotics, as enhancers of the activity of conventional antibiotics and lysozyme, and as anti-endotoxins. Such peptides kill bacteria rapidly, are effective against antibiotic resistant bacteria, and do not easily select resistant mutants. Therefore, we have been designing novel improved peptides. Cationic peptides from all four structural classes have been used as templates and a combination of random and defined alterations, assisted by molecular modelling, biochemical and structural studies, have been introduced. These studies have resulted in molecules with excellent activities, and the potential to become a clinically-significant tool in the fight against resistant bacteria.

1645 Enhancing the inhibitory effects of natural antimicrobial peptides by using combination approaches

PETER J. COOTE[1], C.D. HOLYOAK[2], D. BRACEY[2], D. FERDINANDO[2] and J.A. PEARCE[2]
[1]Centre for Biomolecular Sciences, School of Biology, University of St. Andrews, The North Haugh, St. Andrews, Fife KY16 9ST; [2]Microbiology and Measurement Science, Unilever Research Colworth, Sharnbrook, Bedford MK44 1LQ

Abstract not received

POSTERS

Mi2 01 β-defensin expression in the ovine respiratory epithelium

G. McLACHLAN[1], H. ROWE[2], A. PEMBERTON[3], L. HYNDMAN[1], C.J. DOHERTY[4] and J.R.W. GOVAN[3]
[1]Wellcome Trust Centre for Research in Comparative Respiratory Medicine, [2]Moredun Research Institute, Pentlands Science Park, Edinburgh, [3] Dept of Veterinary Clinical Studies, Easter Bush Veterinary Centre, Midlothian, [4] Dept of Medical Microbiology, University of Edinburgh

Defensins are a family of small, cationic peptides with broad-spectrum antimicrobial activity. It is becoming apparent that these peptides play a significant role in innate immunity not only via their direct antimicrobial activities but also through other interactions with components of inflammatory/immune processes. The β-defensins are expressed by epithelial cells are thought to contribute to the defence system of mucosal surfaces. Two β-defensin sequences, SBD-1 and SBD-2, have been isolated in sheep and shown to be expressed in a variety of epithelial tissues. We are interested in the role of β-defensins in the ovine respiratory epithelium in the normal state and during bacterial infections. We have looked at expression of SBD-1 and SBD-2 by RT-PCR in ovine tracheal ring organ cultures and differentiated ovine tracheal epithelial cell cultures grown at an air/liquid interface either untreated or following challenge with bacteria, TNFα or LPS. SBD-1 is expressed all untreated samples whereas SBD-2 is only expressed at very low levels in a small number of untreated samples. Preliminary results from challenged samples suggest no dramatic up-regulation of expression of these peptides although SBD-2 can be detected in a higher proportion of challenged samples. The sequences of the mature active SBD-1 and SBD-2 molecules and their antimicrobial activities have yet to be characterised. We have identified HPLC fractions from extracts of ovine tracheal epithelium, which exhibit antimicrobial activity. Gel electrophoresis of these fractions

reveal that there are several bands present in the samples with the strongest staining bands migrating between 3-14 KD. Further purification steps are required to isolate and identify the factor(s) present in these fractions which are responsible for the antimicrobial activity.

Mi2 02 The interaction of secretory leukocyte proteinase inhibitor (SLPI) and elafin (elastase-specific inhibitor) with bacterial endotoxin

JONATHAN W. McMICHAEL and JEAN-MICHEL SALLENAVE
Rayne Laboratory, Centre for Inflammation Research, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG

Secretory leukocyte proteinase inhibitor (SLPI) and elafin (elastase-specific inhibitor) are two low molecular weight elastase inhibitors secreted locally in the respiratory tract. Historically, the anti-proteinase activity of SLPI and elafin has been considered to be their primary role, preventing proteolytic damage to the lung by neutralising human neutrophil elastase released extracellularly. However other important functions, such as antimicrobial activity against gram-positive and gram-negative bacteria, have also recently been described.

In this study we have investigated the interaction of SLPI and elafin with bacterial endotoxin (or LPS), the major pro-inflammatory component of gram-negative bacteria. Peptides were incubated overnight at 37°C with LPS of *E. coli* O15 in PBS/1mM EDTA, and run in 15% native polyacrylamide gels at 80V to study direct binding to LPS. In addition, an ELISA assay was used to investigate potential inhibition of the interaction of LPS with LPS-binding protein (LBP): biotinylated LPS of *E. coli* O55:B5 was incubated for 30 minutes at 37°C in the presence or absence of peptides, and added to LBP pre-bound to an anti-LBP monoclonal antibody. Absorbance read at 490nm gave a direct measure of LPS-LBP interaction. We have shown that recombinant human SLPI (rh-SLPI, mw 11.7kDa), recombinant human elafin (rh-elafin, mw 6kDa) and the amino- and carboxy-terminal domains of elafin (mw 5.17kDa and 4.78kDa respectively) can bind to LPS directly and retard its migration in native PAGE gels. Moreover, the peptides displayed the ability to inhibit the interaction of LPS with LBP, as shown by ELISA. In the range 625nM to 10μM, SLPI caused between 42-74% inhibition, while elafin exhibited 27-63% inhibition. The amino- and carboxy-terminal domains of elafin effected maximal inhibitions at 5μM, of 50% and 68% respectively.

By blocking the first step in the LPS-mediated activation of macrophages, SLPI and elafin may play an important role in down-regulating the inflammatory response in the host. Further characterisation of the roles of these peptides in the innate immune response is the subject of ongoing research in our laboratory.

Mi2 03 The permeability changes of the bacterial envelope in response to treatment with magainin I or II

RONALD A DIXON*, MOHAMAD AL NAZAWI and GRACE ALDERSON
Dept of Biomedical Sciences, University of Bradford - *Present address: Dept of Biological and Food Sciences, Lincoln University LS6 7TS

The magainin group of antibacterial peptides are a group of natural or synthesised insect peptides with interesting activity as synergists against a range of bacterial pathogens. The effects of subinhibitory concentrations of magainin I and II against a single strain of *Escherichia coli* K12 were investigated and a significant increase in the sensitivity of the organism to large molecular weight hydrophobic antibiotics (erythromycin, bacitracin etc) was observed.

In order to explain these changes, biochemical measures of permeability in whole bacterial cells were investigated. The *E.coli* outer membrane (OM) is virtually impermable to lysozyme but addition of magainin I and II resulted in lysis of lysozyme-treated cells observed by a rapid and sustained reduction in absorbance values when monitored spectrophotometrically. Furthermore, *E. coli* OM normally excludes the hydrophobic dye crystal violet but addition of magainins to cultures of bacteria significantly enhanced uptake of the dye. Similar results were obtained with a positive control - the well characterised permeabilising agent (polymyxin B nonapeptide - PMBN) in these experiments. The direct effect of magainin treatment on OM integrity was shown by the release of a specific enzyme marker of OM disruption (β-lactamase) in a marked strain. Neither the magainins or PMBN affected the integrity of the inner membrane as judged by the release of cytosolic galactosidase. These results are consistent with magainins causing significant permeability changes to the E.coli OM although it remains unclear why some antibacterial peptides act as permeabilisers and yet others lyse specific bacterial cells. The possible alternative mechanisms of action of antibacterial peptides will be discussed.

Mi2 04 Antibacterial and cytotoxic activity of synthetic antimicrobial peptides

D.A. DEVINE[1], J. STEWARD[2], J. KEEN[1] and T. BROOKS[2]
[1]University of Leeds, [2]Defence Evaluation and Research Agency Porton Down

To identify candidate molecules for development of antimicrobial therapies against significant bacterial pathogens, with or without development of novel delivery systems, peptides were selected with known or anticipated broad-spectrum antibacterial activity. Whilst a few were well documented, less was known about the activity or cytotoxicity of others. Cecropin A 1-7:melittin 2-9 (CAMEL), cecropin A 1-7:magainin 1-13 (CAMAG), MSI-78, histatin Dhvar4, indolicidin CP.11C, brevinin 1 and clavanin AK and two *de novo* peptides ($KLKLLLKLK\text{-}NH_2$, $KLKLLLLLKLK\text{-}NH_2$) were studied. Broad-spectrum activity was confirmed for each, in agarose diffusion and broth microdilution assays, against Gram-negative (*S. typhimurium*, *E. coli*, *V. cholerae*, *B. cepacia*) and Gram-positive (*S. epidermidis*, *S. aureus*, *L. monocytogenes*, *M. luteus*, *B. cereus*) bacteria. Activities of $KLKLLLLLKLK\text{-}NH_2$ and Dhvar4 were inhibited in broth, although $KLKLLLLLKLK\text{-}NH_2$ affected bacterial morphology at non-inhibitory concentrations. Only Dhvar4 lysed human erythrocytes, at $\geq 3.125 \mu g ml^{-1}$. Perturbation of mammalian cell membranes was also assessed using release of lactate dehydrogenase (LDH) from BHK cells: some release was observed for 5 peptides studied but this did not increase with increasing peptide concentration. MTT assays indicated CAMEL, MSI-78 and $KLKLLLKLK\text{-}NH_2$ reduced viability of BHK cells at $\geq 12.5 \mu g ml^{-1}$, Dhvar4 at $\geq 50 \mu g ml^{-1}$ and CAMAG at $100 \mu g ml^{-1}$. Preliminary studies on intracellular killing and peptide delivery systems will continue using a limited number of peptides selected from the above on the basis of activity, toxicity, ease of synthesis and potential for further design.

THURSDAY 29 MARCH 2001

0915 Biological chemistry of nitric oxide

A.J. THOMSON
University of East Anglia, Norwich
Abstract not received

0950 Production and consumption of nitric oxide in denitrifying bacteria

STUART J. FERGUSON
Dept of Biochemistry, University of Oxford, Oxford OX1 3QU
The complete process of denitrification involves four reduction steps that are catalysed by enzymes that are associated with the respiratory chain system of a bacterium. The steps are nitrate to nitrite, nitrite to nitric oxide, nitric oxide to nitrous oxide and nitrous oxide to dinitrogen.

Until the late 1980s it was still vigorously debated as to whether nitric oxide was an intermediate in this sequence; direct reduction of nitrite to nitrous oxide was favoured by some investigators. One reason for this view was the difficulty of detecting nitric oxide in cultures of denitrifying bacteria. Introduction of sensitive measuring methods and extracellular traps, for example hemoglobin, showed that free nitric oxide is formed but at very low steady level concentrations. Thus nitric oxide consumption by the nitric oxide reductase has to be able to more than match the synthesis of nitric oxide by nitrite reductase. Much has now been learned about the reductases that catalyse these two reactions and the way in which they interact with the respiratory chain. Nitric oxide reductase is a membrane-bound enzyme that belongs to the cytochrome oxidase family of enzymes. A change from specificity for oxygen to specificity for nitric oxide appears to correlate with the presence of an iron atom, rather than a copper, adjacent to a heme group at the active site. Curiously there are two kinds of nitrite reductase, one containing copper at the active site and one containing a specialised d1 heme and known as cytochrome cd1.

Progress in understanding these enzymes will be reviewed as will the presence of certain of the denitrifying bacteria in non-denitrifying organisms.

1025 Characterisation of the *Paracoccus denitrificans* transcription factors FnrP and NNR

MATTHEW I. HUTCHINGS[1], NEIL SHEARER[1], JASON CRACK[2], ANDREW J. THOMSON[2] and STEPHEN SPIRO[1]
[1]School of Biological Sciences, [2]School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ
Paracoccus denitrificans is a soil dwelling member of the α sub-group of the proteobacteria which, during anaerobic growth, can generate energy using N-oxides as terminal electron acceptors in a process known as denitrification. Control of denitrification at the genetic level involves at least two members of the FNR/CRP superfamily of transcription regulators. These are FnrP (an orthologue of *E. coli* FNR, the fumarate and nitrate reductase regulator) and NNR (nitrite and nitric oxide reductase regulator). FnrP activates the *nar* (nitrate reductase) operon in response to anoxia, probably sensed through an iron sulphur cluster, and NNR activates the *nir* (nitrite reductase) and *nor* (nitric oxide reductase) operons in response to nitric oxide, which is sensed via an unknown mechanism. Both proteins recognise the same binding site (the FNR box) at their target promoters in *P. denitrificans* and yet there is no cross talk between them. We have expressed both FnrP and NNR in an *E.coli fnr lac* strain and used an artificial FNR-dependent promoter, fused to *lacZ*, to measure the activity of these proteins under various growth conditions. Strikingly, NNR is activated by the NO^+ releasing agent SNP during anaerobic growth, while FnrP is activated by anoxia and not affected by SNP. The NO donors GSNO and SNAP also had similar effects, to varying degrees, on both proteins *in vivo*. We have used this system to characterise a number of *nnr* and *fnrP* mutants, which allows some important conclusions to be drawn about the activity and activation signals of both proteins. We have also purified both transcription factors from GST fusion proteins and carried out biochemical analysis *in vitro*. This analysis includes the reconstitution of an oxygen sensitive iron sulphur cluster into FnrP.

1110 Flavohaemoglobins: proteins with no function?

ROBERT K. POOLE[1], HUGO CRUZ-RAMOS[1], CATHERINE MILLS[1], GUANGHUI WU[1], TANIA STEVANIN[1], MALINI COOPAMAH[1] and MARTIN N. HUGHES[2]
[1]Krebs Institute for Biomolecular Research, Dept of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, [2]Chemistry Dept, King's College London, Strand, London WC2R 2LS
Nitric oxide (NO) is a signalling and defence molecule of major importance, reacting with diverse biological targets to give species with modified activity and toxicity. A recent explosion of interest has triggered an understanding of how bacteria resist NO and related species. The best understood protective mechanism is inducible synthesis of flavohaemoglobins, comprising a haem domain homologous to classical globins and a ferredoxin-$NADP^+$ reductase (FNR)-like domain that converts the globin into an NAD(P)H-oxidising protein with multiple reductase activities. In *Escherichia coli*, the prototype flavohaemoglobin (Hmp) is clearly involved in responses to NO and nitrosative stress, since: (1) the structural gene hmp is markedly up-regulated by NO and nitrosating agents; (2) purified Hmp binds NO avidly but also converts it to nitrate (aerobically) or nitrous oxide (anaerobically); (3) hmp mutants are hypersensitive to NO and nitrosative stresses. Regulation of hmp transcription by NO and related species is complex, but involves at least modulation of MetR function at the hmp promoter by homocysteine pool sizes and disruption by NO of Fe-S clusters in the global regulator, Fnr. Current studies on the enzymic mechanism of Hmp reveal a remarkably peroxidase-like active site, tailored to perform NO and oxygen chemistry.
(This work was supported by BBSRC.)

1145 Response of photosynthetic bacteria to nitric oxide and its derivatives

JAMES P. SHAPLEIGH
Dept of Microbiology, Cornell University, USA
Rhodobacter sphaeroides strain 2.4.3 is a denitrifying bacterium capable of reducing nitrate to nitrous oxide. Nitric oxide is an obligatory intermediate in this process. To mitigate the toxicity of nitric oxide, denitrifiers such as *R. sphaeroides* coordinately regulate the expression of the enzyme that produces nitric oxide, nitrite reductase, and the expression of the enzyme that consumes nitric oxide, nitric oxide reductase, to ensure that the steady state concentrations of nitric oxide stay in the low nM range during denitrification. The regulatory factor responsible for regulating nitrite and nitric oxide reductase activity in *R. sphaeroides* is NnrR. NnrR appears to directly sense nitric oxide although the mechanism by which it does so is not clear. Site-directed mutagenesis of NnrR has identified several residues essential for NnrR activity. The related photosynthetic bacterium, *Rhodopseudomonas palustris*, encodes two nitrite reductases and an NnrR with significant

similarity to NnrR from 2.4.3. One of the two nitrite reductases appears to be regulated by NnrR since it can be expressed in *R. sphaeroides* 2.4.3 strain grown in nitrate amended medium. Unexpectedly, there is no expression of the same fusion in *R. palustris*, even in the presence of the inducer sodium nitroprusside. Experiments are currently underway to investigate why NnrR in *R. palustris* appears to be inactive. A related strain of *R. sphaeroides*, strain 2.4.1, is naturally nitrite reductase deficient, even though it contains NnrR and nitric oxide reductase. We have found this strain is also naturally more sensitive to nitric oxide modified thiols than the 2.4.3 strain. We have begun mutagenesis of the 2.4.3 strain to identify genes involved in increasing tolerance to nitrosothiols. Five mutants have been isolated that are more sensitive to S-nitrosoglutathione than the parent strain. Preliminary evidence indicates three of the mutants have insertions in DNA regions that are not present in the 2.4.1 strain, while one of the remaining two mutants is in the operon encoding nitric oxide reductase. We have also found that a strain of 2.4.1 expressing nitrite reductase becomes more resistant to nitrosothiols. Experiments are currently underway to characterize the genes in 2.4.3 whose inactivation increases nitrosothiol sensitivity.

1330 Overview of nitric oxide metabolism in healthy and infected cells

T. EVANS
Imperial College School of Medicine, London
Abstract not received

1405 NO signalling, no response: the *soxRS* system

BRUCE DEMPLE, HUANGEN DING, ANASTASIA KOUTSOLIOUTSOU, PABLO POMPOSIELLO, MARGARET JORGENSEN and MONICA CHANDER
Dept of Cancer Cell Biology, Harvard School of Public Health, Boston, Massachusetts 02115, USA
In *Escherichia coli*, the *soxRS* system modulates ~50 genes that contribute to defenses against free radical damage and other functions. The sensor that governs this response, SoxR, is a transcription activator that contains [2Fe-2S] iron-sulfur centers. These metal centers are in the reduced state in inactive SoxR; they are oxidized during superoxide stress, which causes strong stimulation of *soxS* transcription. SoxS protein then activates the many genes of the *soxRS* regulon. The *soxRS* system is also activated during phagocytosis of *E. coli* by murine peritoneal macrophages, in an NO-dependent fashion. This activation can be reproduced by treating intact bacteria with pure NO, and EPR spectroscopy shows that a new form of SoxR is generated with properties identical to mixed dithiol-iron-dinitrosyl complexes. The formation and removal of the modified complexes (when NO is depleted) parallels the transcription of the *soxS* gene exactly. The same NO-modified form of SoxR was generated *in vitro* by treating the pure protein with pure NO. Nitrosylated SoxR was stable enough to be repurified, with transcriptional activity similar to that of oxidized SoxR. Since nitrosylation inactivates the iron-sulfur centers of many enzymes in all cell types, the rapid turnover of nitrosylated SoxR in cells may reflect an important protein repair process.

1440 Nitric oxide and circulatory shock: from benchside to bedside

CHRIS THIEMERMANN
Dept of Experimental Medicine & Nephrology, William Harvey Research Institute, St. Bartholomew's and The Royal London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ
Nitric oxide (NO) is generated by three different isoforms of NO synthase, two of which are expressed constitutively (in endothelium: eNOS, brain: nNOS), while one is induced by endotoxin or cytokines (iNOS). Expression of iNOS in many organs/tissues in septic shock results in an enhanced formation of NO, which contributes to circulatory collapse and possibly host defence. Inhibition of NOS activity in shock has beneficial and adverse effects, which have tentatively been linked to inhibition of iNOS and eNOS activity, respectively. The non-selective NOS inhibitor L-NMMA exerts beneficial haemodynamic effects in animals and patients with septic shock. In a phase II clinical trial, administration of L-NMMA (for 72 h) in 156 patients with septic shock caused resolution of shock and reduced the need for vasopressor support. A recent phase III clinical trial, in which L-NMMA was administered to patients with septic shock (up to 20 mg/kg/h for up to 14 days), was stopped due to safety concerns (e.g. a trend for an increase in mortality). One reported side effect of L-NMMA in animals and man with septic shock is a further rise in pulmonary vascular resistance. There is evidence (in pigs with severe endotoxaemia) that NO-gas inhalation exerts beneficial haemodynamic effects and attenuates the rise in pulmonary artery pressure caused by L-NMMA. Although inhibition of iNOS activity attenuates the circulatory failure in endotoxin (or septic) shock in many species, it is less clear whether selective iNOS inhibitors reduce the organ injury or mortality associated with shock. We have recently reported that two highly selective inhibitors of iNOS activity, namely 1400W and L-NIL, abolish the formation of NO by iNOS and the circulatory failure without reducing the degree of organ injury/dysfunction caused by endotoxin in animals. Studies subjecting mice in which the iNOS gene has been inactivated by gene-targeting to endotoxaemia support the notion that NO from iNOS contributes to hypotension and host defence, but not to the liver injury caused by endotoxin. Thus, inhibition of iNOS activity appears to reduce the circulatory failure, but not the multiple organ failure associated with septic shock.

1545 Antimicrobial actions of NO

FERRIC C. FANG, JEFFREY SCHAPIRO, JESSICA JONES-CARSON and ANDRÉS VAZQUEZ-TORRES
Depts of Medicine, Pathology and Microbiology, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, B168, Denver, Colorado 80262, USA - Tel: 303-315-4857, Fax: 303-315-8681, Email: ferric.fang@uchsc.edu
Nitrogen oxides produced by phagocytic cells have been strongly implicated as important antimicrobial mediators against parasites, fungi, bacteria and viruses. Both humans and experimental animals produce dramatically increased quantities of nitric oxide (NO) during infection, and expression of NO synthase can be directly demonstrated in infected tissues. Inhibition of NO synthase exacerbates microbial proliferation during experimental infections or phagocyte killing assays, and a variety of chemical NO-donors have been shown to inhibit or kill diverse microbial species in vitro. Nitrogen oxides can act both at the microbial surface and within the cell, oxidizing membrane lipids, directly or indirectly modifying DNA, and reacting with protein thiols, metal centers, amines and aromatic residues. Microbes can employ a variety of defensive strategies against nitrogen oxides, including avoidance, expression of stress regulons, direct or indirect detoxification, scavenging, repair of nitrosative damage and inhibition of production. An improved understanding of NO targets and relevant mechanisms of resistance will shed new light on microbial pathogenesis and lead to the discovery and application of new therapies for infectious diseases.

1620 NO Resistance in *Neisseria meningitidis*

TANIA STEVANIN, MUNA ANJUM, LINDA GOODWIN, JAMES MOIR and ROBERT READ
Dept of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN
In order to colonise and cause disease, the human pathogen *Neisseria meningitidis* withstands the human host's immune response, including the production of nitric oxide (NO). The meningococcal genome contains (at least) two genes that may have a role in conferring resistance to NO; namely *cycP* encoding cytochrome *c*' and *norB* encoding a single-subunit NO reductase. Translational *lacZ* fusions have revealed that

both genes are expressed maximally under conditions when NO is being synthesised by denitrification of nitrite. *cycP* mutants are impaired in their capacity to respire oxygen when challenged with NO. Growth by denitrification requires the presence of an intact *norB* gene. The capacity of the meningococcus to survive in culture with macrophages and in nasal explant tissue culture, and the role of NO protective mechanisms in these models will be discussed.

1635 Nitric oxide as a signal in plant disease resistance

C. LAMB
John Innes Centre, Norwich
Abstract not received

POSTERS

PBMG 01 Nitric oxide metabolism in the freshwater cyanobacterium ***Synechocystis*** PCC6803

KERRIE ALLEN-O'ROURKE and STEPHEN SPIRO
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ
The genome of the freshwater cyanobacterium *Synechocystis* PCC6803 includes a gene designated *norB* that potentially encodes a nitric oxide reductase (NOR), of the type thought to accept electrons from the quinone pool. Divergently transcribed from *norB* is a gene (*dnr*) encoding a protein related to NNR of *Paracoccus denitrificans* that activates NOR expression in response to nitric oxide. There is a potential DNR binding site in the *norB-dnr* intergenic region, suggesting that, in *Synechocystis*, the *dnr* gene product might regulate *norB* expression in response to nitric oxide. Chromosomal mutations have been introduced into the *norB* and *dnr* genes, in order to explore the physiological role of nitric oxide reduction in this organism. Results of reporter fusion and mRNA analyses will be presented, to illustrate the function of DNR in regulating expression of *norB*.

PBMG 02 Characterisation of the *Paracoccus denitrificans* transcription factors FnrP and NNR

MATTHEW I. HUTCHINGS[1], NEIL SHEARER[1], JASON CRACK[2], ANDREW J. THOMSON[2] and STEPHEN SPIRO[1]
[1]School of Biological Sciences and [2]School of Chemical Sciences, University of East Anglia, Norwich NR4 7TJ
Paracoccus denitrificans is a soil dwelling member of the α sub-group of the proteobacteria which, during anaerobic growth, can generate energy using N-oxides as terminal electron acceptors in a process known as denitrification. Control of denitrification at the genetic level involves at least two members of the FNR/CRP superfamily of transcription regulators. These are FnrP (an orthologue of *E. coli* FNR, the fumarate and nitrate reductase regulator) and NNR (nitrite and nitric oxide reductase regulator). FnrP activates the *nar* (nitrate reductase) operon in response to anoxia, probably sensed through an iron sulphur cluster, and NNR activates the *nir* (nitrite reductase) and *nor* (nitric oxide reductase) operons in response to nitric oxide, which is sensed via an unknown mechanism. Both proteins recognise the same binding site (the FNR box) at their target promoters in *P. denitrificans* and yet there is no cross talk between them. We have expressed both FnrP and NNR in an *E.coli fnr lac* strain and used an artificial FNR-dependent promoter, fused to *lacZ*, to measure the activity of these proteins under various growth conditions. Strikingly, NNR is activated by the NO^+ releasing agent SNP during anaerobic growth, while FnrP is activated by anoxia and not affected by SNP. The NO donors GSNO and SNAP also had similar effects, to varying degrees, on both proteins *in vivo*. We have used this system to characterise a number of *nnr* and *fnrP* mutants, which allows some important conclusions to be drawn about the activity and activation signals of both proteins. We have also purified both transcription factors from GST fusion proteins and carried out biochemical analysis *in vitro*. This analysis includes the reconstitution of an oxygen sensitive iron sulphur cluster into FnrP.

PBMG 03 General enhanced synthesis of carbon-energy-source transport systems during adaptation of *E. coli* to growth at low glucose concentrations

LUKAS M. WICK[1], MANFREDO QUADRONI[2+] and THOMAS EGLI[1]
[1]Swiss Federal Institute for Environmental Science and Technology (EAWAG), P.O. Box 611, CH-8600 Dübendorf, Switzerland, [2]Protein Chemistry Laboratory, Swiss Federal Institute of Technology Zürich, CH-8092 Zürich, Switzerland - [+]Present address: Institute of Biochemistry, University of Lausanne, CH-1066 Epalinges, Switzerland
The adaptation from high to low nutrient concentrations, and *vice versa*, is an important process in the life cycle of micro-organisms having to cope with conditions in which sometimes oligotrophic sometimes copiotrophic properties are required for optimum survival. Therefore, we investigated the events that allow this adaptation using *E. coli* K12 MG1655 as a model organism.

Adaptation to low substrate concentration was investigated by transferring *E. coli* cells from nutrient-excess batch growth to glucose-limited continuous culture. It was shown before that the residual glucose concentration in the cultivation liquid decreases in a hyperbolic-like fashion. In order to investigate what changes in protein expression occur when cells adapt to the glucose-limited conditions, two-dimensional gel electrophoresis analysis was performed. Samples were taken from batch grown culture and from the continuous cultivation 40 and 500 hours after the start of the continuous cultivation. By this analysis changes were detected which occurred in the short term during transition from glucose-excess batch to glucose-limited continuous culture and on the other hand changes of a long-term adaptation type to these glucose-limited conditions.

The transition from batch to continuous culture led in a short term to a stronger expression of several different transport proteins. Although grown on glucose minimal medium these proteins include also transporters not related to glucose transport. The long term adaptation during 500 hours in the continuous cultivation resulted in a higher expression of transport proteins which have a high affinity to glucose.

This suggests that on the short term the cells respond to nutrient limitation by a general effort in substrate scavenging. And that on the long term they adapt (by selection) specifically to the conditions applied (glucose-limited conditions in this case).

PBMG 04 The kinetics of adaptation of *E. coli* to low glucose concentrations in continuous culture

LUKAS M. WICK and THOMAS EGLI
Swiss Federal Institute for Environmental Science and Technology (EAWAG), P.O. Box 611, CH-8600 Dübendorf, Switzerland
Several studies performed with a number of different bacteria have reported strong evidence for a significant improvement of glucose affinity as a result of long-term cultivation at low glucose concentration. *E. coli* cells cultivated in continuous culture for several weeks show mutations in and over-expression of specific genes which lead to higher glucose affinity as shown before by genetic and proteomic studies (see other poster by the same authors).

In this work the kinetics of this adaptation process in two *E. coli* strains has been investigated, based on the determination of the residual glucose concentration in continuous cultures. In all experiments the residual glucose concentration in the culture decreased in a hyperbolic-like fashion after the culture had been switched to the continuous growth mode. The time required until the residual concentration of glucose became constant was in the order of 300 to 400 hours, independent of the tested dilution rates (0.1, 0.2, and $0.3h^{-1}$). During this time the apparent K_s improved from 400 to 40 mg/l and the evolution of glucose

concentration with time was absolutely reproducible between independent runs. This implies that at lower dilution rates it takes fewer generations to adapt to glucose-limited condition. A mathematical model was used to explain this behaviour and to predict the evolution of the residual glucose concentration as a function of cultivation time. The model is based on the Monod equation for microbial growth and assumes a stepwise improvement of the K_s with a certain mutation rate.

In the case of the dilution rate of $0.1h^{-1}$ the K_s in the first 80 hours was higher than expected from the model. It is possible that in this case the expression of RpoS, which is known to become induced at low growth rates, is influencing adaptation kinetics.

PBMG 05 *Escherichia coli* flavohaemoglobin, Hmp, is a NO-Detoxifying globin with a peroxidase-like catalytic site

MASAHIRO MUKAI[1], CATHERINE E. MILLS[2], ROBERT K. POOLE[2] and SYUN-RU YEH[1]
[1]Dept of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461, USA, and [2]Dept of Molecular Biology and Biotechnology, The University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN

The flavohaemoglobin (Hmp) in *E. coli* is an NO-inducible protein essential for resistance to NO and nitrosative stress. Instead of oxygen delivery, this globin acts aerobically as a dioxygenase, converting NO to nitrate, and anaerobically as a NO reductase, converting NO to N_2O. To investigate the structural features underlying the reactivity of Hmp, we have measured the resonance Raman spectra of the ligand-free ferric and ferrous protein and the CO derivatives of the ferrous protein. At neutral pH, like peroxidases, the ferric protein has a five-coordinate high-spin haem. In the ferrous protein, a strong iron-histidine stretching mode is present at 244 cm^{-1}. This frequency is much higher than that of any other known globin, but is comparable to those of peroxidases, suggesting that the proximal histidine has imidazolate character. In the CO derivative, an open and a closed conformation were detected. The distal environment of the closed conformation is very polar, in which the haem-bound CO strongly interacts with the B10 Tyr and/or the E7 Gln. These data demonstrate that the active site structure of Hmp is very similar to that of peroxidases and is tailored to perform oxygen chemistry. *(This work was supported by BBSRC and NIH.)*

PBMG 06 Nitric oxide sensing by the *Escherichia coli* transcriptional regulator, FNR: role of its iron-sulfur cluster and DNA-binding activity in expression of the *hmp* (flavohaemoglobin) gene

HUGO CRUZ-RAMOS, GUANGHUI WU and ROBERT K. POOLE
Krebs Institute for Biomolecular Research, Dept of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Sheffield S10 2TN

Transcription of the *Escherichia coli hmp* gene is controlled in part by FNR, which senses O_2 by assembly-disassembly of a $[4Fe\text{-}4S]^{2+}$ cluster. In the active $[4Fe\text{-}4S]^{2+}$ form, FNR dimerisation and subsequent site-specific DNA binding increase. In view of interactions between other iron-sulfur proteins and NO, we investigated the possibility that FNR senses NO. In purified FNR, NO elicits loss of the 420 nm absorbance of the $[4Fe\text{-}4S]^{2+}$ cluster. The disrupted Fe-S cluster is reconstructed by a standard reconstitution treatment, suggesting a physiological significance. In band shift assays, reconstituted FNR retards a fragment of the *hmp* promoter containing the FNR site centred at the +5.5 position. Specificity of the anaerobic DNA shifting was confirmed by point mutations within this FNR-binding site. Interestingly, FNR samples exposed to either O_2 or NO retain some ability to bind the promoter. DNA binding activity has been studied by DNase I footprint and biochemical analyses. *In vivo* also, NO regulates expression of FNR-regulated genes in a manner consistent with disruption of the $[4Fe\text{-}4S]^{2+}$ cluster. Thus, the *fnr* regulon can be modulated, not only by O_2, but also by NO or related nitrosative stress conditions. We propose an NO-mediated mechanism of anaerobic up-regulation of the *hmp* gene by which *E. coli* responds to nitrosative stress. *(This work was supported by BBSRC.)*

PBMG 07 *Escherichia co*li flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of NO

CATHERINE E. MILLS[1], SVETA SEDELNIKOVA[1], MARTIN N. HUGHES[2] and ROBERT K. POOLE[1]
[1]Krebs Institute for Biomolecular Research, Dept of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Sheffield S10 2TN, [2]Dept of Chemistry, King's College London, Strand, London WC2R 2LS

The *E. coli* flavohaemoglobin Hmp comprises an N-terminal globin-like haem domain and an FAD-binding reductase domain. We have developed a purification that gives high yields of protein with equistoichiometric redox centres. NADH oxidation by the purified protein generates H_2O_2, which presumably arises from superoxide, also detectable during oxygen reduction; water is not a product. In the absence of agents that scavenge superoxide and peroxide, the K_m for oxygen is around 80 µM; addition of 15 µM FAD decreases the K_m for oxygen to 15 µM without change in V_{max}, but also catalyses cyanide-insensitive oxygen consumption, attributed to electron transfer from flavins to O_2. In the absence of added FAD, purified Hmp consumes NO (approx. 1 O_2/NO) consistent with NO oxygenation. However, half-maximal rates of NO-stimulated O_2 consumption requires about 50 µM O_2, and NO removal is ineffective below about 30 µM O_2. Cyanide (100 µM) abolishes NO oxygenase activity of purified Hmp. On exhaustion of O_2, NO (36 µM) is removed by a cyanide-sensitive process attributed to NO reduction, with a turnover about one per cent of that for oxygenase activity. These results suggest that the ability of Hmp to detoxify NO may be compromised in hypoxic environments. *(This work was supported by BBSRC.)*

PBMG 08 Upstream regulation of actII-ORF4, a key activator of antibiotic production in *Streptomyces coelicolor*

K. JOLLY, K.J. MCDOWALL and S. BAUMBERG
School of Biology, University of Leeds, Clarendon Way, Leeds LS2 9JT

Streptomycetes (filamentous soil bacteria) produce antibiotics in response to environmental triggers. The genes required for antibiotic biosynthesis are clustered and their expression is almost invariably activated by a pathway specific protein; for example, actinorhodin biosynthesis in *S. coelicolor* is activated by production of ActII-ORF4, and streptomycin biosynthesis in *S. griseus* by StrR. These particular pathway specific regulators belong to different regulatory protein families. To elucidate the regulatory system that links environmental changes to the production of ActII-ORF4, a DNA-binding assay has been used to identify proteins that bind around the promoter region of *actII-ORF4* and are therefore candidate transcriptional regulators of its expression. We have detected two DNA-binding activities, one of which binds specifically to two separate regions flanking the transcriptional start point of *actII-ORF4*. Interestingly, this specific DNA-binding protein also binds to the upstream region of the *S. griseus* gene that encodes StrR. This suggests that the mechanisms regulating the production of antibiotic pathway specific activators in disparate streptomycetes may be evolutionarily conserved, even though the final activators differ substantially.

PBMG 09 Establishment of gene transfer to *Lactococcus lactis* IO-1

A.J. STEELE and D.R. WILLIAMS
Dept of Biosciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB

Lactococcus lactis IO-1 is capable of the fermentation of xylose to produce lactic acid with high yield. As such it could utilise the xylose present in acid digests of agricultural wastes rich in hemicelluloses, such as palm oil waste. However, the presence of glucose in these wastes prevents the co-utilisation of the sugars due to catabolite repression. We have investigated the efficiencies of different methods of gene transfer to this strain. The techniques used include inter-species conjugation from *Escherichia coli* and direct transformation by electroporation. The establishment of efficient gene transfer allows the subsequent development of gene disruption techniques and the alteration of gene dosage. Manipulation of the strain in these ways may allow the construction of strains where catabolite repression has been relieved and the sugars in waste hydrolysates may be co-utilised with the production of lactic acid.

PBMG 10 The feasibility of resistance plasmid destabilisation in the treatment of infections

N.J. CREWE and D.R. WILLIAMS
Dept of Biosciences, University of Hertfordshire, College Lane, Hatfield AL10 9AB

Antibiotic resistance is becoming an increasingly large burden in the treatment of clinical infections. Resistance genes are often carried on large low copy number plasmids that contain stability systems to ensure they persist in the population. We are undertaking a feasibility study to determine whether reduction of antibiotic resistance due to plasmid destabilisation indicates that stability systems are a potential target for enhancing the treatment of infections by some organisms. Use has been made of a group of genetically altered plasmids with differing levels of stability. Chemostat culture has been used to assess stability from the size of the plasmid free population in the presence of antibiotics. It has also been used to measure the effect of destabilisation on growth rates in antibiotic containing environments, treating the chemostat as a simplified model of a clinical infection.

MONDAY 26 MARCH 2001

0910 Whither Genomics? - I Beg to Differ

J. EISEN
TIGR, 9712 Medical Center Drive, Rockville, MD 20850, USA

I review here the current state of complete genome sequencing summarizing some of the lessons that have been learned from the some 40 complete genome sequences that are now available. I will focus in particular on the question that many have been asking recently - whether the era of complete genome sequencing is over and that we need to now move on to "post-genomics". I will show that the currently available and in progress genome projects represent but a tiny fraction of diversity at different levels including evolution, ecology, physiology, biochemistry, and behavior. I will also show that these genome projects are incredibly limited in regard to economic, medical, and agricultural importance. Finally, I present recent findings from multiple research groups that show that the scientific value of a complete genome sequence is much greater than a partial genome sequence. These and other lines of evidence show that, while many more questions are created by complete genome sequences than are answered, we have by no means reached the saturation point of the value of complete genome sequencing.

0950 Estimating rates of recombination in bacterial pathogens, and the impact of recombination, from DNA sequences

BRIAN G. SPRATT
Wellcome Trust Centre for the Epidemiology of Infectious Disease, Oxford University, Oxford OX1 3FY

Bacteria divide by binary fission and are often thought of as asexual organisms. However, genetic mechanisms that can lead to the replacement of small regions of the chromosome with the corresponding regions from other isolates are well known. The extent to which these recombinational exchange mechanisms are used in nature, and their impact on the evolution of bacterial populations, has been controversial. The existence of clones, and of linkage disequilibrium between the alleles at different loci, has often been taken as evidence for a low rate of recombination within natural populations of bacteria. However, clones and linkage disequilibrium can be present within populations in which localised recombinational exchanges are relatively common, and a more critical and quantitative approach is required to understand the extent and impact of recombination in different bacterial species. In recent years, the nucleotide sequences of internal fragments of seven house-keeping genes, from hundreds of isolates of different bacterial species, have become available from the introduction of multilocus sequence typing (MLST) as the gold standard for the unambiguous characterisation of bacterial isolates for epidemiological studies. These sequences can be used both to quantitate the contribution of recombination, compared to point mutation, in the diversification of bacterial clones, and to examine the longer-term impact of recombination on the population. In many bacterial species, evolutionary change at neutral (house-keeping) loci has been shown to be much more commonly brought about by recombination than by point mutation and, in several of these species, recombination has been sufficiently frequent to result in net-like evolution, and to preclude attempts to reconstruct the true evolutionary relationships between lineages of the species.

1100 ORF distribution among prokaryotes: vertical and lateral themes

MARK RAGAN
Institute for Molecular Bioscience, The University of Queensland, Australia

If open reading frames (ORFs) have been transmitted primarily by vertical descent within lineages, sets of orthologs should be distributed among genomes so as to form patterns consistent with a single common organismal tree. Distributional patterns not parsimoniously reconciled with treelike descent are *prima facie* evidence for lateral gene transfer (LGT). The clearest patterns can be found by setting different criteria for presence and absence: BLASTP matches better than a stringent threshold are probable orthologs, while the lack of a match above a more-permissive threshold is evidence that homologs are absent, or much less conserved (*i.e.*, probable paralogs). ORFs in bacterial genomes usually exhibit a bimodal distribution, finding matches either in all other bacterial phyla, or in only one; the latter tend not to be with sister phyla, but with those most-represented in GenBank. These ORFs were examined for other evidence of LGT, including atypical base compositions, prediction by atypical hidden Markov models, and atypical orders of BLASTP matches. These different approaches to detecting LGT find the same ORFs less often than expected by chance. Bacterial ORFs can thus be atypical in different ways, and atypicality *per se* does not necessarily imply lateral origin. Returning to fundamentals, a comprehensive approach to identifying LGT in microbial genomes will be outlined.

1140 Selfish Operon Model - Tracing genomic flux, gene clustering and the evolution of genotype and its impact on phenotype

JEFFREY LAWRENCE
Dept of Biological Sciences, 352 Crawford Hall, University of Pittsburgh, Pittsburgh, PA 15260, USA

Unlike crown eukaryotic species, microbial species are created by continual processes of gene loss and acquisition promoted by horizontal genetic transfer. The amounts of foreign DNA in bacterial genomes, and the rate at which it's acquired, are consistent with gene transfer as the primary catalyst for microbial differentiation. In addition, chronic horizontal gene transfer results in the inevitable clustering of genes into broad-host-range cassettes that can confer complex metabolic phenotypes upon acquisition. However, the rate of successful gene transfer varies among bacterial lineages. The heterogeneity in foreign DNA content is directly correlated with amount of genetic headroom intrinsic to a bacterial species. Genetic headroom reflects the amount of potentially dispensable information reflected in codon usage bias and codon context bias that can be transiently sacrificed to allow experimentation with functions introduced by gene transfer. Moreover, large genetic headroom reduces the necessary selective benefit acquired genes must confer to allow long-term persistence. I describe novel methods to quantitate the amount of genetic headroom evident in a microbial genome sequences, and use this measure to predict rates of horizontal gene transfer. In this way, genetic headroom offers potential metric for the propensity of a lineage to speciate.

1400 A shifted paradigm: impact of lateral gene transfer on the origin and diversification of eukaryotes

LAURA A. KATZ
Dept of Biological Sciences, Smith College, Northampton, MA, USA (Graduate program in Organismic and Evolutionary Biology, University of Massachusetts, MA USA)

Vertical transmission of heritable material, a cornerstone of the Darwinian theory of evolution, is inadequate to describe the evolution of eukaryotes. This is because eukaryotic genomes are chimeric, having evolved through a combination of vertical (parent to offspring) and lateral (trans-species) transmission. To understand the origin and diversification of eukaryotic lineages we must now elucidate the tempo and mode of lateral transfer events. Moreover, we must disentangle factors including the number of donor lineages, the nature of what was transferred and the relationship between donor and recipient. Comprehensive answers to these questions require complete genome sequences from a number of diverse eukaryotes and prokaryotes combined with sequences of targeted genes from a broader phylogenetic sample. As these data continue to emerge, we can describe the gene transfer events that have impacted extant eukaryotes as well as provide insight into lineages that once inhabited the earth.

1440 *Rickettsia, Bartonella* and the origin of Mitochondria

SIV G.E. ANDERSSON, O. KARLBERG, B. CANBÄCK and C.G. KURLAND
Dept of Molecular Evolution, Evolutionary Biology Center, University of Uppsala, Uppsala, Sweden

The endosymbiotic theory for the origin of mitochondria requires substantial modification. Here, a novel scheme for the origin of mitochondria based on phylogenetic reconstructions of several hundred mitochondrial proteins will be discussed. Some mitochondrial proteins are clearly of alpha proteobacterial origin, as inferred from their close sequence relationships to genes in modern representatives of the alpha proteobacteria, such as *Rickettsia* and *Bartonella*. This set of proteins represents core components of the bioenergetic system and the protein synthesis machinery in mitochondria. Others have no homology to bacterial proteins and were presumably recruited from the nuclear genome subsequent to the acquisition of mitochondria so as to complement the genes that were transferred from the bacterial endosymbiont. Many of these secondarily derived proteins are involved in fine-tuning the communication process between the nucleus and the mitochondrion. We believe that these eukaryotic genes transformed the initial oxygen-consuming endosymbiont into an ATP-exporting organelle.

1540 Phylogeny and genomic history of "Early Eukaryotes"

T.M. EMBLEY
The Natural History Museum, London

Until recently our ideas about early eukaryote evolution were largely based upon characters mapped over the small subunit ribosomal RNA gene tree. One particularly attractive idea - the Archezoa Hypothesis - was that some eukaryotes, including microsporidia, Giardia and Trichomonas, separated from other eukaryotes before the mitochondrion symbiosis. Thus the origin of the eukaryotic nucleus preceded the acquisition of mitochondria and the earliest eukaryotes were anaerobic. However, recent data and more critical analyses have cast doubt on the ability of 18S rRNA gene sequences to resolve basal branching pattern and have also, perhaps decisively, damaged the Archezoa Hypothesis. I will review problems and recent progress towards producing a more robust phylogeny for eukaryotes including the central challenge of finding the root of the eukaryotic tree. I will also discuss some recent analyses of genomic data for *Trichomonas vaginalis* and Spironucleus - a distant relative of Giardia - which bear on these issues and also on the evolution of parasitism in these particular taxa.

1620 Impact of genomic data on our understanding of the origin of eukaryotic cells

W. MARTIN
Institut fuer Botanik III, Heinrich-Heine Universitaet Duesseldorf, Universitaetsstr. 1, 40225 Duesseldorf, Germany

In order to estimate the fraction of Arabidopsis genes that come from cyanobacteria, we examined 368 phylogenetic trees filtered from 3961 protein coding genes and 18 reference genomes. Our criterion for scoring an Arabidopsis gene as stemming from cyanobacteria is simple - we asked "is the Arabidopsis protein more similar to its cyanobacterial homologue than it is to homologues from any other reference genome?" Translated into the language of phylogenetic trees, that means asking "does the Arabidopsis protein share a common branch with its cyanobacterial homologue in a protein phylogeny?" Using that criterion in protein maximum likelihood trees and using simple likelihood ratio tests, we found that between 1.6% and 9.2% of the Arabidopsis genes examined are likely to be acqusitions from cyanobacteria. But we also found evidence to suggest that the differing levels of sequence conservation among proteins may be the primary factor limiting this estimate. Some of the premisses involved in inferring eukaryotic gene origins will be outlined, also as it applies to the origins of genes acquired from mitochondria (in addition to those inherited from the host). The issue of how lateral gene transfer between free-living prokaryotes complicates these matters will be discussed.

TUESDAY 27 MARCH 2001

0900 The role of horizontal gene transfer in genome evolution

JAMES LAKE, RAVI JAIN and MARIA RIVERA
Molecular Biology Institute and MCD Biology, University of California, Los Angeles, CA 90095

Increasingly, studies of genes and genomes are indicating that considerable horizontal transfer has occurred even between distantly related prokaryotes. In a phylogenetic analysis of six complete prokaryotic genomes, containing some 12,000 genes, we find that horizontal gene transfer is infrequent for genes comprising the translational and transcriptional apparatuses (informational genes) but that horizontal transfer is extensive for operational genes (those involved in housekeeping). When horizontal gene transfer is analyzed by following 300+ orthologous genes common to six genomes from six organisms broadly representative of prokaryotic diversity, we find that operational genes have been horizontally transferred in an ongoing process operating continuously since the divergence of the prokaryotes.

Since rates of evolution differ little between operational and informational genes, factors other than rate of evolution are responsible for the resistance of translational genes to horizontal transfer. Our studies suggest that a major cause of the infrequent horizontal transfer of translational genes, as well as other informational genes, is that they are typically members of large complex systems, whereas operational genes are not. Thus it is less likely that informational genes will function well, once transferred to a new host, since they must make multiple contacts to function, whereas operational genes require few contacts to function (the Complexity Hypothesis).

0940 Prokaryotic expression monitoring by differential display and microarray

JAMES T. FLEMING
Center for Environmental Biotechnology, University of Tennessee, 676 Dabney Hall, Knoxville, TN, USA

Genome-wide screening methods offer great potential for the study of microbial processes both in pure culture and microbial communities. We have adapted the complimentary procedures of differential display (DD) and microarray analysis to the study of prokaryotic gene expression.

DD permits the isolation of differentially expressed genes under inducing conditions from complex biological systems without the prior culturing of organisms or prior sequence information. The method is, therefore, well suited for the study of complex uncharacterized environmental or industrial microbial processes with mixed or unknown populations. A DD method with modifications for application to prokaryotes has been used to clone differentially expressed genes from pure cultures, consortia and soil microcosms.

While microarray analysis, because of the requirement for prior sequence information, is more immediately applicable to sequenced organisms compared to uncharacterized communities, several projects are currently underway with the goal of applying array base methods to environmental analysis. In a study of uranium induction of *Escherischia coli* triplicate experiments using all 4920 expressed genes were analyzed using several different statistical models, the value of which are presently being evaluated using sub-arrays. In another project microarrays created with representative clones from rRNA intergenic libraries obtained from industrial wastewater populations are being used to deduce community structure.

1100 Prokaryote polymorphism and the SENTINEL project

BARRY ROBSON
Strategic Advisor, IBM Research, T.J. Watson Research Centre, USA

Selected mutations leading to rich polymorphism in prokaryote genomes are long understood to be an important aspect of prokaryotic evolution and human health-care. In pathogens generally, they are responsible for the phenomenon of "escape from restriction" from animal and specific human populations, to become epidemics, as well as being an important factor in the appearance of drug resistance. The SENTINEL project is a venture into "immuno-informatics" comprising an early phase study of a global web-based early warning and defense system against natural and, potentially, military aggression. A primary feature is a pathogen genome and proteome database with emphasis on prediction of both continuous and discontinuous epitopes for the production of diagnostic-biosensor arrays, which would be input devices to the network. The overall system is essentially auto-catalytic because detection in changes to the spectrum of response across an array of diverse monoclonals would lead to identification of new data and the development of new monoclonal entries on those arrays. Also, because the "raising of antibodies" is a step both in diagnostic and vaccine development, the diagnsotic-biosesnsor sensor arrays are intended to lead to rapid development of cartridge vaccines, i.e. vaccines with synthetic peptide "plug-in" components representing B-epitopes, T-epitope and Cytotxoic T-cell components, immunostimulatory components and so on.

The speaker was some years ago the founder of the SENTINEL concept and in the late 1960s involved with the early studies of mutations in the pencillinases (beta-lactamases) of *S. Aureus* and their effect of the folding, function and inhibition of the enzyme, later the development of vaccine design software, the synthesis and testing of diagnostics and cartridge vaccines, and recently the early phase planning of the SENTINEL informatics infrastructure.

1140 Genome spatial information system (GenoSIS)

K. BEARD
National Center for Geographic Information and Analysis, University of Maine, USA

Abstract not received

1400 Comparative analysis of bacterial pathogens

J. PARKHILL
Sanger Centre, Cambridge

Abstract not received

1440 Transcriptional profile signatures as tools for understanding host-pathogen interactions and evolution of virulence traits

STEPHEN LORY
Dept of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA

Infections of immunocompromized humans by certain common bacterial pathogens provide an opportunity to analyze genome-wide responses when the bacteria change their habitat from their natural environment to human tissues. The interaction of the opportunistic pathogen *Pseudomonas aeruginosa,* with epithelial cells and human respiratory mucus, was investigated using expression microarrays of human and bacterial genes. Using isogenic pairs of wild type and mutant *P. aeruginosa* strains defective in individual virulence factor genes, transcriptional profiling in epithelial cells was able to identify unique sets of human genes which are activated or expressed in response to specific bacterial products. The expression of a particular bacterial product provides a diagnostic signature for the presence of the infecting bacterium in the host. Similarly, the transcriptional response in *P. aeruginosa,* grown under conditions, which mimic the human respiratory tract, was investigated using a *P. aeruginosa* genomic microarray. The expression of a number of metabolic, housekeeping and most significantly, specific virulence factor genes was altered under different environmental conditions, including those during exposure to human respiratory mucus. The global gene expression in bacteria is a sensitive method for determining the makeup of complex environmental signals, which are essential for successful colonization of the host during infection.

1540 What can functional genomics tell us about the biology of bioremediation?

CAROLINE S. HARWOOD
University of Iowa, USA

Bacterial pathways for the degradation of various environmental pollutants, such as trichloroethylene, toluene, and naphthalene, have been studied in detail. Generally degradation sequences that contribute to bioremediation are studied one pathway at a time in isolation. A model bacterium that is a good degrader of the pollutant in question provides the raw material for bioremediation studies. This approach has been tremendously useful, but it does suffer from limitations. One of which is that we still have limited knowledge about biological rules that govern the degradation of mixtures of pollutants. Also, we don't have a good idea of the degree to which ancillary biological processes contribute to biodegradation. Such processes include transport, chemotaxis, solvent tolerance, and surfactant production. In short we have a poor understanding of bioremedation processes as they occur in the context of the 'biology" of entire microbes. This is about to change. In this current era of microbial genomics it is now technically possible to take a comprehensive look at the complete biodegradation potential of an individual microbial species. Techniques of gene expression profiling and proteomics can be used to determine how the expression of sets of genes that are required for the degradation of a particular compound is modulated depending on the quantities of other pollutants that the bacterium is exposed to. Similarly it is possible to see how the processes of chemotaxis, transport and solvent tolerance may operate in the microcosm of a single microbial species to enhance its bioremediation potential. These concepts will be discussed in the context of the genome sequences of the bacteria *Rhodopseudomonas palustris* and *Pseudomonas putida*.

1620 The *Streptomyces violaceoruber* clade as a model for defining taxonomic relationships at the species level

ALAN C. WARD, KANNIKA DUANGMAL, GAIL PAYNE, ROS BROWN, ERNST LACEY, WON Y. KIM and MICHAEL GOODFELLOW
Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU

The *Streptomyces coelicolor* A3(2) genome sequence is virtually complete and is the model genome for streptomycetes. This taxonomically diverse, morphologically and metabolically complex, and ecologically widespread group of organisms has been widely isolated and cultured, is taxonomically well-studied and their molecular biology, genetics and physiology has been extensively researched. Nevertheless their metabolic diversity poses challenges in understanding their taxonomy, evolution, soil ecology and industrial application. The extent to which the *S. coelicolor* A3(2) genome can be utilised as a model depends upon the evolutionary divergence across the clade.

S. coelicolor A3(2) is a member of cluster 21 *sensu* Williams *et al*. (1983) and part of the *S. violaceoruber* clade, as defined by 16S rDNA sequencing. Using an existing phenotypic database, selective isolation media for cluster 21 strains has been devised and strains isolated from diverse habitats. The whole genome sequence data of *S. coelicolor* A3(2) and f-AFLP of type strains and new isolates in the *S. violaceoruber* clade has been used to compare phenotype and genotype in this well-defined species group.

1635 Genomics and the biodegradation of nitroaromatic compounds

REBECCA E. PARALES
Dept of Microbiology, The University of Iowa, USA

Nitroaromatic compounds are rarely produced by biological processes. Consequently, the vast majority of nitroaromatic compounds present in the environment are due to the last century of industrial activity. Bacteria that are capable of utilizing nitroarene compounds such as nitrobenzene, 2-nitrotoluene and 2,4-, and 2,6-dinitrotoluenes as sole carbon and nitrogen sources have recently been isolated. The first step in the degradation of these compounds is catalyzed by multicomponent Rieske non-heme iron dioxygenases. Genes encoding nitroarene dioxygenases have been cloned from several strains and based on sequence analyses, all appear to have been recruited from a naphthalene degradation pathway. Nearly identical sets of nitroarene dioxygenase genes have been identified in the genomes of distantly related organisms. Genes encoding consecutive steps in nitroarene degradation pathways are frequently unlinked and are not coordinately regulated. In several instances, sequences with similarities to transposase genes or insertion elements have been found near nitroarene degradation genes, suggesting that horizontal transfer of these genes occurred during pathway evolution. In addition, vestigial genes with no known role in nitroarene degradation are located within gene clusters encoding nitroarene dioxygenases. These results indicate that nitroarene degradation pathways have evolved very recently and are not yet optimized.

1650 Genomes may provide insight into microbial evolution and bacterial speciation

J.T. STALEY, C. JENKINS, B.P. HEDLUND and J.A. DODSWORTH
University of Washington, Seattle, WA USA 98195, USA

Most scientists believe that the Bacteria and Archaea were the first organisms on Earth based on their simple morphology and diverse metabolisms, physiologies and phylogeny. If so, their genomes will provide much of what can be inferred molecularly about the early evolution of life. For this reason it is important that a genome from at least one representative of each of the prokaryotic phyla be sequenced to allow for comparative analyses. These genomes will provide insight into the overall patterns of biological evolution as well as more specific processes such as bacterial speciation. Two examples will be presented to illustrate the utility of bacterial genome sequencing. The first example involves the genome of *Prosthecobacter vanneervenii*, a member of the division Verrucomicrobia. Results from this project, in progress with Integrated Genomics Incorporated, will be presented and discussed. The other example pertains more specifically to the process of bacterial speciation. The commensal bacterial genus, *Simonsiella*, lives in the oral cavity of mammals. Questions that will be discussed are (a) Can the study of commensals provide knowledge about bacterial speciation? (b) Can the genome sequences of commensals be used to identify the genes that are responsible for determining the niche of a species?

POSTERS

SE 01 Taxonomic evaluation of the Streptomyces violaceus species-group

G. PAYNE[1], G.P. MANFIO[2], J. ZAKRZEWSKA-CZERWINSKA[3] and M. GOODFELLOW[1]
[1]Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU; [2]Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland, [3]Tropical Culture Collection, Fundação Tropical Pesquisas e Technologia, Campinas, Brazil

Extensive numerical phenetic surveys led to the assignment of validly described species of Streptomyces to minor and single membered clusters which were equated with species and to major clusters that were considered as species-groups. Where possible, the numerically defined taxa were named after the earliest described species they contained. In the present study, the eight strains classified in the Stre*ptomyces violaceus* species-group were examined using a number of molecular systematic procedures. The DNA:DNA relatedness data showed that all of the strains belong to different genomic species. The organisms also gave distinct ribotype patterns and Rep-PCR fingerprints and were distinguished using a combination of phenotypic properties and by Curie point pyrolysis mass spectrometry. The genotypic and phenotypic data indicate that *S. cellostaticus*, *S. michiganensis*, *S. showdoensis*, *S. spiroverticillatus*, *S. venezuelae*, *S. vinaceus* and *S. violescens* should be retained as validly described species and should not be seen as synonyms or close relatives of *S. violaceus*. These results provide further evidence of the dangers of delineating streptomycete species solely on the basis of numerical taxonomic data. It is clear that with taxonomically complex groups such as the genus Streptomyces only the polyphasic approach can be expected to yield well defined species and a stable nomenclature.

SE 02 Characterisation of Staurosporine-producing Streptomycetes

ROS BROWN[1], ERNEST LACEY[2], STUART ROBINSON[2] and MICHAEL GOODFELLOW[1]
[1]Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU, [2]Microbial Screening Technologies, Yarrandoo Research Station, Kemps Creek, NSW, Australia, 2171

Members of the genus Streptomyces have a unique ability to produce commercially significant bioactive compounds, notably antibiotics. As part of a screening programme designed to detect novel metabolites of interest three putatively novel streptomycetes were found to produce staurosporine, an anti-cancer compound first isolated from a strain named Saccharothrix aerocolonigenes subsp *staurosporeus*. All three strains were found to have chemical and morphological properties consistent with their classification in the genus Streptomyces. Nearly complete 16S rDNA sequences of the organisms were compared with

corresponding sequences of available type strains of the genus Streptomyces that were retrieved from the RDP database. The resulting data were analysed using several treeing algorithms; the three organisms formed a novel and distinct clade within the evolutionary radiation encompassed by members of the genus Streptomyces. The organisms were also distinguished from phylogenetically related strains using a range of phenotypic properties. The genotypic and phenotypic data indicate that the three organisms belong to a new species of Streptomyces.

SE 03 Characterisation of novel members of the genus *Nonomuraea*

ERIKA QUINTANA, MARTHA E. TRUJILLO, LUIS MALDONADO and MICHAEL GOODFELLOW
Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU

The emergence of antibiotic resistant pathogens has led to a search for rare and novel actinomycetes which may prove to be the source of new and effective therapeutic natural products, including antibiotics. In a search for such novel actinomycetes from soil samples five organisms were isolated and provisionally assigned to the genus Nonomuraea. This taxon currently encompasses sixteen validly described species which form a distinct phyletic line within the evolutionary radiation encompassed by members of the family Streptosporangiaceae. Almost complete 16S rDNA sequences of the five strains were compared with corresponding sequences of representatives of the family Streptosporangiaceae. It was evident from the resultant phylogenetic tree that all five strains formed new centres of taxonomic variation within the evolutionary radiation occupied by the genus Nonomuraea. The taxonomic status of the five strains was also underpinned by additional genotypic and phenotypic data.

SE 04 Selective isolation of Amycolatopsis strains from environmental samples using antimicrobial agents

GEOK YUAN ANNIE TAN, ALAN C. WARD and MICHAEL GOODFELLOW
Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU

The genus Amycolatopsis contains eleven validly described species which form a distinct phyletic line within the evolutionary radiation encompassed by the family Pseudonocardiaceae. Members of the genus are a rich source of bioactive compounds which include the commercially significant antibiotics, rifamycin and vancomycin. The search and discovery of additional therapeutic compounds is hampered by the difficulty of isolating members of the genus from natural habitats, notably soil. In the present study, large numbers of Amycolatopsis-like colonies were isolated following the incubation of soil suspensions on several media supplemented with antimicrobial agents. Representatives of these isolates were found to have chemical and morphological properties typical of Amycolatopsis strains. Nearly complete 16S rDNA sequences of several of these isolates were compared with corresponding sequences of the type strains of the validly described species of Amycolatopsis. The resultant data were analysed using several treeing algorithms; all of the isolates formed distinct phyletic lines within the Amycolatopsis tree. The taxonomic integrity of these strains was underpinned by additional genotypic and phenotypic data.

SE 05 Selective isolation and characterisation of members of the *Streptomyces violaceoruber clade*

KANNIKA DUANGMAL, ALAN C. WARD and MICHAEL GOODFELLOW
Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU

Streptomycetes have a unique capacity to produce bioactive compounds, though novel approaches are now needed for the selective isolation of representatives of new species for pharmaceutical screening programmes. The primary aim of the present study was to design media selective for members of the Streptomyces *violaceoruber* clade, which includes *S. coelicolor* A3(2). Isolates with morphological and pigmentation properties characteristic of members of this clade, such as " *S. caesius*", *S. coelescens*, "*S. coelicolor*", *S. humiferus*, "*S. lividans*", *S. violaceolatus and S. violaceoruber*, were detected on selective isolation media inoculated with suspensions prepared from several composite soil samples. One hundred and fifty representative isolates were assigned to four artificial colour groups based on pigments produced on oatmeal and peptone-yeast extract iron agars. Almost complete 16S rDNA sequences prepared from representatives of the colour groups were compared with available corresponding sequences of representatives of the genus Streptomyces retrieved from the RDP database. All of the representative strains were assigned to the *S. violaceoruber* clade. The finer taxonomic relationships of the representative isolates were established using various molecular techniques, including Rep-PCR, AFLP and DNA:DNA pairing. The systematic isolation of many isolates from a well defined clade and comparison *with S. coelicolorA* 3(2) will allow the extent of new variation with currently defined streptomycete diversity to be determined.

SE 06 Comparative analysis of a small fragment of the rpoB gene as a means of identifying Nocardia strains

LUIS MALDONADO and MICHAEL GOODFELLOW
Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU

The genus Nocardia has had a long and tortuous taxonomic history primarily due to the overemphasis placed on morphological criteria. However, the genus currently encompasses twenty-one validly described species the status of which is underpinned by a wealth of genotypic and phenotypic data. The revised classification provides a sound framework for the recognition of additional species though improved methods are needed for the rapid and reliable assignment of unknown nocardiae to validly described and putatively novel species. With this in mind the sequences of a fragment of the RNA β subunit gene, rpoB, were determined for representative nocardiae and the data analysed using four treeing algorithms. The representatives of both recognised and putatively novel species formed distinct phyletic lines in the resultant tree. The taxonomic integrity of the phyletic lines was supported by corresponding 16S rDNA sequence and phenotypic data. It can be concluded, therefore, that the rpoB sequencing procedure provides a quick and accurate way of assigning unknown nocardiae, isolated from clinical and environmental sources, to recognised and prospective novel species of Nocardia.

SE 07 Genetic structure of *Salmonella enterica*

FIONA SCOTT and CATH ARNOLD
Molecular Biology Unit, Virus Reference Division, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT

To elucidate the genetic structure of *Salmonella enterica*, the three Salmonella reference collections (SARA, SARB and SARC) were analysed by fluorescent amplified fragment length polymorphism (FAFLP). These collections represent 160 strains of 52 serotypes from the seven subspecies of *Salmonella enterica*. Multilocus enzyme electrophoresis (MLEE) has previously been used to establish the basic population genetic framework of Salmonella and to analyse inter and intra serotype variation, and MLEE studies have demonstrated the basically clonal structure of natural populations of Salmonella by comparing the relative electrophoretic mobilities of a small number of metabolic enzymes. For FAFLP, genomic DNA was digested with the restriction enzymes MseI and EcoRI. Oligonucleotide adapters were ligated and amplification of specific subsets of fragments was performed using the selective primer combination Eco+O and Mse+TA. Fragments were separated on a sequencing gel, fragment presence and absence was

scored for each strain, and distance matrices were calculated. Trees were constructed in the Neighbour-Joining programme of Phylip. FAFLP analysis of 156 SARA, SARB and SARC reference strains yielded 142 distinct FAFLP patterns (there were 14 strain duplications between the collections). Several distinct clusters of isolates were observed. The 16 serotypes that comprise SARC fell into 8 discrete clusters that corresponded precisely with the subspecies of Salmonella. Two strains of SARB that were indistinguishable by MLEE were found to be separate but closely related by FAFLP. The 11 serotypes that were polyphyletic by MLEE were also found to be polyphyletic by FAFLP. In SARA several distinct FAFLP clusters of isolates were observed that correspond to the clusters observed by MLEE. FAFLP, an accurate and robust molecular typing technique, supports the taxonomic grouping of Salmonella based on DNA hybridisation studies, and confirms and extends published.

SE 08 Characterisation of a new actinomycete species that degrades methyl t-butyl ether

AMANDA JONES[1], JOSEPH P. SALANITRO[2] and MICHAEL GOODFELLOW[1]
[1]Dept of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne NE1 7RU, [2]Equilon Enterprises LLC, Westhollow Technology Centre, Houston, Texas 77251-1380, USA

Alkyl ethers such as methyl t-butyl ether (MTBE) are used as octane enhancers in the reformulation of low-volatility unleaded gasoline blends and for reducing emissions of volatile organic compounds from engines. There is little information on the biodegradability of MTBE in soil, groundwater, and activated sludge systems. The taxonomic position of two MTBE-degrading actinomycetes was determined in the present study using a combination of genotypic and phenotypic properties. The organisms were found to have phenotypic properties typical of members of the genus *Rhodococcus* and were assigned to the 16S rDNA subclade which encompasses *Rhodococcus rhodochrous* and closely related species. The two strains share many phenotypic characters in common and belong to the same genomic species albeit one sharply separated from *Rhodococcus ruber* with which they form a distinct phyletic line. The organisms were also distinguished from all of the species classified in the *R. rhodochrous* subgroup using a combination of phenotypic properties. The genotypic and phenotypic data show that the two strains merit recognition as a new species of *Rhodococcus*.

WEDNESDAY 28 MARCH 2001

0900 The multifunctional herpes simplex virus ICP27 protein

J. BARKLIE CLEMENTS[1], MARIA KOFFA[1], HELEN BRYANT[1], SARAH WADD[1], STUART WILSON[2], ELISA IZZURALDE[3] and IAIN MATTAJ[3]
[1]Division of Virology, Institute of Biomedical and Life Sciences, University of Glasgow, Church Street, Glasgow, G11 5J, [2]Dept of Biomolecular Sciences UMIST, PO Box 88, Manchester M60 1QD, [3]EMBL. Meyerhofstrasse 1, 69117, Heidelberg, Germany

Herpes simplex virus (HSV-1) is a double stranded nuclear replicating virus. Two HSV-1 regulatory proteins are essential for viral growth and one of these, ICP27 (IE63), is the only HSV-1 immediate early gene with a counterpart in all sequenced herpesviruses of mammalian and avian origin. Studies of this protein are important to key questions about herpesvirus biology.

ICP27 is a multifunctional protein that regulates gene expression at transcriptional and post-transcriptional levels (reviewed in 1). HSV expresses some 80 transcripts during lytic infection and only four undergo splicing, the remainder being intronless. ICP27 inhibits host cell splicing, shuttles from nucleus to cytoplasm and binds intronless viral RNAs but does not bind intron containing HSV transcripts which accumulate in nuclear clumps (2), suggesting the protein mediates nuclear export of intronless viral RNAs (3).

Nuclear export of ICP27 is blocked by the drug leptomycin B (LMB) indicating a requirement of the export receptor CRM-1 for its nuclear exit. Intriguingly, treatment with LMB blocks the accumulation of certain HSV RNAs and proteins but not all of them (4).

Our recent data show that ICP27 interacts with a constitutive splicing factor, SAP145, that splicing is inhibited before the first step in catalysis and ICP27 is detected co-migrating with the splicing complexes formed. ICP27 interacts with the ubiquitous casein kinase 2 (CK2), hnRNP K (5) and REF-2, an hnRNP-like protein. Early in HSV infection, CK2 activity is upregulated by activating the α'-subunit and CK2 holoenzyme redistributes from nucleus to the cytoplasm: export of IPC27 is important for both these effects. In turn, activated CK2 phosphorylates ICP27 and promotes its export as well as phosphorylating hnRNP K which is not ordinarily a CK2 substrate.

REF participates in mRNA nuclear export through interaction with TAP, probably facilitating the interaction of TAP with cellular mRNAs (6). Although TAP does not seem to mediate cellular mRNA export by binding to mRNA, it binds to a constitutive transport element (CTE) of the simian type D retroviruses and promotes CTE-dependent export from the nucleus (7). When RNA from the late Us11 HSV gene, which is responsive to LMB treatment, was injected into Xenopus oocytes in the presence or absence of injected recombinant ICP27 we found that ICP27 dramatically stimulated the export of Us11 RNA without affecting export of cellular mRNA, U snRNA or tRNA. TAP protein further stimulated the export of viral RNA, while an excess of CTE RNA saturated its export.

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0945 Cis-acting negative RNA elements on papillomavirus late mRNAs

STEFAN SCHWARTZ, MARCUS SOKOLOWSKI, BRIAN COLLIER, DANIEL ÖBERG, MARGARET RUSH and ANETTE CARLSSON
Dept of Medical Biochemistry and Microbiology, BMC, Uppsala University, Sweden

Human papillomaviruses (HPVs) may be divided into groups of types that produce high or low levels of HPV virions in vivo or into those that are cancer-associated and persist for longer periods of time and those that are benign and normally persist for a relatively short time period in the host. We have selected one highly productive, low risk HPV type (HPV-1) and a high risk HPV type with low virus production (HPV-16) for our studies. Although they differ in the abundance of virions detected in vivo, they both show a cell-differentiation dependent production of the late structural proteins L1 and L2 and that was the focus of our research. Two types of negative regulatory RNA elements have been identified on late HPV mRNAs to date: elements in the 3' UTR and negative elements in the L1 and L2 coding regions on the late mRNAs. The former are present in both HPV-1 and HPV-16, and their presence therefore correlates with the differentiation dependent L1 and L2 production observed in cells infected with these virus types. The negative elements in the L1 and L2 coding regions are present in HPV-16, but not in HPV-1. Their existence therefore correlates with low virus production and the ability of the virus to persist for longer periods in the infected host. This is of particular interest since persistence of high-risk HPV types is a risk factor for development of cervical cancer. The identification and characterisation of the negative RNA elements will be presented.

1100 Post-transcriptional regulation of influenza virus gene expression

JUAN ORTIN, ROSA M. MARION, TOMAS ARAGON, ANA M. FALCON, PURI FORTES, SUSANA DE LA LUNA, ANA BELOSO, ISABEL NOVOA, LUIS CARRASCO, CARLOS DOTTI and AMELIA NIETO
Centro Nacional de Biotecnologia (CSIC0, Cantoblanco, 28049 Madrid Spain, Centro de Biologia Molecular (CSIC-UAM), Cantoblanco, 28049 Madrid, Spain and European Molecular Biology Laboratory, Meyerhofstr. 1, 69012 Heidelberg, Germany

Influenza virus NS1 protein is an RNA-binding protein whose expression in mammalian cells alters various cellular post-transcriptional processes. Thus, it inhibits pre-mRNA polyadenylation and splicing and mRNA nucleo-cytoplasmic transport. In addition, NS1 protein stimulates translation of viral mRNAs. To investigate the mechanisms responsible for these effects, we have looked for cellular proteins able to interact with NS1 protein by co-immunoprecipitation experiments and two-hybrid screening with yeast.

We have found that NS1 protein co-immunoprecipitates with translation initiation factor 4GI, the large subunit of eIF4F, in cotransfected cells and in influenza virus-infected cells. Purified NS1 protein could pull down 4GI protein but not the 4E subunit of eIF4F. Mapping studies using 4GI deletion mutants indicated that the NS1 binding domain is located between positions 157 and 550 in 4GI protein, a region where no other component of the translation machinery is known to interact. On the other hand, the N-termina 113 amino acids of NS1 protein are sufficient for interaction with 4GI protein. Such a deleted protein had been previously shown to enhance translation of influenza virus mRNAs in vivo. Collectively, these data suggest that NS1

protein recruits 4GI factor specifically to ciral mRNAs and allows for their preferential translation in infected cells.

As a result of a two-hybrid screen with NS1 protein as a bait, ahuman cDNA clone was identified capable of coding for a protein with high homology to the staufen proten from D. melanogaster (dmStaufen). The encoded protein (hStaufen) contained 4 dsRNA binding domains with 38% identity to those of dmStaufen, including identity at all residues involved in RNA binding. A recombinant protein containing all dsRNA binding domains, expressed in E. coli as a His-tagged polypeptide, showed dsRNA binding activity in vitro. Using a specific antibody, a main form of the hStaufen protein could be detected in human cells, with apparent molecular mass of 60-65 kDa. The intracellular localization of hStaufen protein was investigated by immunofluorescence, using a series of markers for the cell compartments. Co-localization was observed with rough endoplasmic reticulum, but not with endosomes, cytoskeletion or Golgi apparatus. Furthermore, sedimentation analyses indicated that hStaufen protein associates with polysomes. The interaction of hStaufen with NS1 protein was studied both in civo and in vitro. Both proteins could be co-immunoprecipitated from influenza virus-infected cells, when co-expressed in cultured cells or when mixed in vitro. Furthermore, hStaufen and NS1 proteins co-fractionate in the polysomes of influenza virus-infected cells.

To gain information about the role of hStaufen protein in cultured human cells and the possible implication of its association to NS1 in the influenza virus infection, we set out to identify RNAs and proteins that associate to hStaufen in human cells. To that aim we have expressed a tagged hStaufen protein by transfection. Such a tagged protein localized intracellularly in a manner indistinguishable from endogenous wt hStaufen. The purification of this recombinant hStaufen and the identification of the attached RNAs and proteins is in progess.

1145 Post-transcriptional RNA processing during hepatitis delta virus replication

JOHN TAYLOR, GLORIA MORALEDA, SEVERIN GUDIMA and JINHONG CHANG
Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA

Hepatitis delta virus (HDV) is natural sub-viral satellite of the human hepadnavirus, hepatitis B virus. The genome of HDV is a small single-stranded RNA (1,679 nt.) with a circular conformation. Transcription of the genome is via RNA-directed RNA synthesis, which is considered to involve redirection of one or more host DNA-directed RNA polymerases. Three HDV RNA species are readily detected during such replication: (i) the genomic RNA circle, (ii) an exact complement, the antigenome, and (iii) a less than unit-length antigenomic polyadenylated RNA that acts as mRNA for the only protein encoded by HDV, the delta antigen (195 a.a.). During replication the nascent RNA transcripts are subjected to a large variety of forms of post-transcriptional processing that includes: (i) specific cleavages by two ribozymes, (ii) specific non-ribozyme cleavage, (iii) 5'-capping, (iv) 3'-poly(A)-processing, and (v) editing by a double-stranded RNA-activated adenosine deaminase at some sites, of which one site is essential. For the replication to be successful there not only has to be regulation of these processes, but some of the RNAs must fuel additional rounds of RNA transcription and processing, and ultimately, in the presence of the helper hepadnavirus, achieve assembly and release of new infectious particles.

1400 Regulation of retroviral gene expression at the level of nuclear mRNA export

BRYAN R. CULLEN
Howard Hughes Medical Institute at Duke University Medical Center, Durham, NC 27710, USA

All retroviruses must express both spliced and unspliced forms of their initial, genome length transcript. Retroviruses have therefore had to develop ways to deal with cellular factors that normally prevent the nuclear export of mRNAs that retain intact introns. At least two pathways have evolved in different retroviral families to deal with this problem. Lentiviruses such as HIV-1, as well as the HTLV-I family and the endogenous HERV-K group, encode a viral regulatory protein, termed Rev in HIV-1, that recruits the cellular nuclear export factor Crm1 to a cis-acting target present in incompletely spliced viral mRNAs. Surprisingly, Crm1 has no known role in cellular mRNA export and instead normally acts to export snRNAs and a variety of proteins from the nucleus. In contrast, several simple retroviruses have evolved viral RNA targets, termed CTEs, that directly recruit a distinct cellular factor, termed Tap, that also plays a key role in cellular mRNA export. However, Tap binding to cellular mRNAs is normally tightly regulated such that only mature mRNAs can recruit Tap. The CTE circumvents this regulation by direct recruitment of Tap to the unspliced viral RNA. I will review the current understanding of both Rev and Tap dependent nuclear RNA export.

1445 Studies on the influence of defective influenza virus RNAs post-transfection on WSN influenza virus generated entirely from plasmids

S.D. DUHAUT and N.J. DIMMOCK
Dept of Biological Sciences, University of Warwick, Coventry CV4 7AL

Previous work on influenza virus defective RNAs established that they can compete with the segment from which they were derived in RNA packaging. Also that internal sequence has a major influence on the stability of defective RNAs in tissue culture, such that increasing amounts of the 5' end of vRNA increase stability. This effect is independent of virus subtype, cell type used, segment of origin or size of defective RNA.

WSN virus plasmids were transfected into Vero cells with increasing amounts of an avian influenza defective plasmid. Virus titres were reduced by one to two logs at 24 and 48 hours post-transfection at the highest amount of defective RNA added, but the effect could be diluted out.

WSN virus plasmids were then transfected into Vero cells with defective equine plasmids possessing increasing amounts of the 5' end of vRNA. The plasmids were all derived from segment 1 and were identical in size. Virus titres were again reduced by one to two logs at 24 and 48 hours post-transfection.

This work suggests that internal sequences of influenza virus defective RNAs towards the 5' end are competing with the virus genomic RNAs at some essential step.

1500 The role of influenza A virus proteins M1 and M2 in virus assembly

D. CHAPMAN[1], C. ELLEMAN[1], R. HARVEY[1], P. DIGARD[2], T. ZURCHER[3] and W. BARCLAY[1]
[1]University of Reading, [2]Cambridge University, and [3]Glaxo-Wellcome Medicines Research Centre, Stevenage

We have been studying virion formation by influenza A virus. In particular we note that certain strains of the H3N2 subtype form protrusions from the infected cells surface and generate filamentous virus particles. Using a reverse genetics approach we have demonstrated that this phenotype is encoded by RNA segment 7. Two proteins are encoded from this segment, the matrix protein M1 and a small transmembrane protein with ion channel properties, M2. The role of each of these gene products in virus assembly, as well as the effect of host cell genes in this process will be discussed.

1515 Polarised intranuclear distibution of the influenza virus nucleoprotein

P. DIGARD, L. MEDCALF, M. SIMPSON-HOLLEY and D. ELTON
Division of Virology, Dept of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP

Polarization of mammalian cells is an established concept, often viewed as compartmentalization of the apical and

basolateral surfaces of the plasma membrane. Many viruses specifically target these membrane domains during entry and/or exit of the host cell. Influenza virus utilises the apical surface for virus assembly and the viral glycoproteins are known to contain apical targetting signals. However, virus transcription occurs in the nucleus of infected cells. The genomic RNA segments are encapsidated by the viral NP to form ribonucleoprotein structures which are later exported from the nucleus and assembled into progeny virions at the plasma membrane. In a striking parallel with the localisation of the viral glycoproteins, NP adopts an apically polarised distribution within the nucleus of infected cells. Initial observations suggest that this phenomenon is not dependent on other influenza polypeptides as exogenously expressed NP shows similar intranuclear localisation. This distribution of NP is therefore presumably due to interactions with cellular components and we are attempting to identify these. We find no significant colocalisation with components of the nuclear import or export machinery, but partial colocalisation with acetylated histones H3 and H4. To our knowledge, this is the first description of intranuclear polarity for a viral polypeptide.

1600 DNA array analysis of murine gammaherpes virus 68 transcriptional profiles

J.W. AHN, D. ALBER and K. POWELL
Wolfson Institute for Biomedical Research, University College London, Cruciform Building, Gower Street, London, WC1E 6AU

Murine gammaherpesvirus-68 (MHV-68) has been shown to be an amenable animal model for the gammaherpesviruses. To characterise the transcriptional profiles of MHV-68, we have developed a novel membrane-based DNA array representing all predicted open reading frames and inter-gene sections. Using this technique, progression through the virus' life cycle has been observed from a transcriptional perspective *in vitro*. Genes were further classified by blocking *de novo* protein synthesis and DNA replication with cycloheximide and 2'-deoxy-5-ethyl-β-4'-thiouridine respectively.

The MHV-68 array allows any changes in gene expression to be rapidly analysed at the genome level. We will present data obtained from the characterisation of two mutants strains examining the knock-on effects of knocking out single genes. This provides insights to the function of the genes in question, as well as their position in the context of global gene expression.

1615 MHC class I ubiquitination by a viral PHD/LAP zinc finger protein

J.M. BONAME and P.G. STEVENSON
Division of Virology, Dept of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP

The murine b-herpesvirus-68 K3 (MK3) is a PHD/LAP zinc finger protein that down-regulates MHC class I expression. A combination of cell lines transfected with wild-type (wt) and mutant MK3 were subjected to radio-immune precipitation analyses to dissect the mechanism of H-2D^b MHC class I down-regulation. Cells expressing wt MK3 showed a rapid degradation of H-2D^b, whereas two mutants with disruptions of the PHD/LAP zinc finger domain did not. The MK3-mediated degradation of H-2D^b was not due to misfolding, nor to a failure of peptide loading. Wt MK3 and its mutant forms were all shown to bind to H-2D^b, thus disruption of the PHD/LAP finger of MK3 did not prevent H-2D^b binding, but did prevent its degradation. When proteasome inhibitors were used, ubiquitinated intermediates of H-2D^b were apparent in cell-lines expressing an intact PHD/LAP finger. The exploitation of normal cellular protein catabolism establishes a novel mechanism of immune evasion, with a viral PHD/LAP zinc finger protein acting as an E3 ubiquitin-protein ligase.

1630 A potential role for the HSV-1 structural protein VP13/14 in gene expression

M. DONNELLY and G. ELLIOT
Marie Curie Research Institute, The Chart, Oxted, Surrey RH8 0TL

The HSV-1 gene UL47 encodes the major structural proteins VP13 and VP14 (VP13/14) which are incorporated into the tegument region of the virus particle. While the role of VP13/14 in virus infection is not yet known, early studies indicated that VP13/14 may modulate the activity of another tegument protein, the transactivator of immediate-early (IE) gene expression VP16. Using live cell analysis of GFP-tagged VP13/14, we have recently shown that VP13/14 localises predominantly to the nucleus when expressed during either transient transfection or virus infection. Furthermore, we have shown that VP13/14 is capable of nucleocytoplasmic shuttling, implying that VP13/14 may play a role in gene expression. Here, we demonstrate that VP13/14 has the ability to transactivate IE gene expression in isolation from VP16. However, we also show that cotransfection of VP13/14 and VP16 results in the efficient relocalisation of VP16 to the nucleus, an interaction which was confirmed by co-immunoprecipitation of VP13/14 with VP16. Taken together these results imply that VP13/14 has two potential roles in virus gene expression - one as a modulator of VP16 activity, and one as a transactivator in its own right.

1645 HSV-1 latency associated promoter driven expression decreases with time after delivery to the rat CNS of replication defective viruses

C. SCARPINI and S. EFSTATHIOU
Dept of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP

HSV-1 Latency Associated Promoter (LAP) drives long-term expression of its endogenous transcripts (known as LATs), as well as of β-Gal in the PNS and in same CNS nuclei. However, it is not known if all CNS neurones can sustain this long-term expression. To target a wide variety of CNS neurones, gH-tk-replication defective viruses were delivered to the rat striatum by stereotaxic injection. Large numbers of neurones expressing LATs or β-Gal under the control of the LAP could be identified up to 5 weeks post delivery at many CNS sites. However, a dramatic decrease in the number of expressing neurones occurred with time at all loci targeted. An immune response characterised by the presence of CD8+ T-cells was observed. This immune response colocalised with LATs and β-Gal expressing neurones and decreased together with the expression from the LAP. However, experiments conducted in nude rats lacking CD8+ T-cells also showed a decrease in b-Gal expressing cells with time. Moreover, studies with a highly disabled HSV-1 vector showed that the loss of β-Gal expressing cells was not due to viral IE gene expression toxicity. Therefore only a restricted population of CNS neurones can sustain long-term expression from the LAP.

1700 Functional analysis of the transmembrane domain of HSV-1 glycoprotein H

A. HARMAN, H. BROWNE and T. MINSON
Division of Virology, Dept of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP

Glycoproteins gB, gD, gH and gL are essential for virus entry and fusion of the viral envelope with the host cell plasma membrane. These proteins are also capable of inducing membrane fusion when expressed in cells from plasmid vectors, although the mechanism buy which membrane fusion occurs is not known. We have investigated the requirement for the transmembrane domain of gH in the fusion process by replacing it with either that of the human CD8 molecule, or with that of influenza haemagglutinin. Both constructs were unable to mediate fusion in the transient fusion system, and both were unable to complement the infectivity of a gH-negative virus. I contrast, a gD molecule containing the CD8 transmembrane domain was functional in fusion and infectivity assays. Site-directed mutagenesis of conserved

residues within the gH transmembrane domain was also carried out, and the effects of these mutations on virus-free fusion and virus entry rates were determined. The results of these studies highlight the requirement for a conserved glycine residue at position 812 in the gH sequence for efficient HSV-1-induced membrane fusion.

1715 Subcellular compartmentalisation of Hepatitis B virus X protein

J. HOARE[1], F. HENKLER[3], R.D. GOLDIN[2], R. KOSHY[4] and M. J McGARVEY
[1]Dept of Medicine, [2]Dept of Histopathology, Imperial College of Science, Technology and Medicine, St. Mary's Campus, London W2 1NY, [3]National Institute for Medical Research, Division of Membrane Biology, Mill Hill, London NW71 AA, [4]National Institutes of Health, Hepatitis Viruses Section, Bethesda, USA. [5] Present Address: Institute for Cell Biology and Immunology, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

The functional mechanisms of the hepatitis B HBx protein are still unclear. Although interactions with several nuclear and cytoplasmic proteins have been demonstrated in vitro, there is no consensus as to where HBx localises in infected hepatocytes. We have analysed the intracellular localisation of HBx, in human liver biopsies and in transfected cells. We demonstrate HBx expression in 70% of biopsies from HBV infected patients with nuclear and cytoplasmic localisation.

In transfected cells we demonstrate that compartmentalisation of HBx is dependent on overall expression levels. HBx was predominantly localised in the nuclei of weakly expressing cells. However, elevated cellular levels correlated with its accumulation in the cytoplasm. We have further analysed cytoplasmic compartmentalisation, using confocal microscopy, and show a substantial association of HBx with mitochondria. However, a major fraction of cytoplasmic HBx did not localize in mitochondria, indicating the presence of two distinctly compartmentalised cytoplasmic populations. Furthermore, high levels of HBx expression led to an abnormal mitochondrial distribution, involving organelle aggregation, not observed at lower expression levels. This data provides novel insights into the compartmentalisation of HBx and may prove important for evaluations of its functions, both in the viral life cycle and the pathology of HBV-related liver disease.

THURSDAY 29 MARCH 2001

0900 Mechanisms of viral IRES-mediated initiation of translation

TATYANA V. PESTOVA[1,2], V.G. KOLUPAEVA[1], I.B. LOMAKIN[1], E.V. PILIPENKO[1], V.I. AGOL[2] and CHRISTOPHER U.T. HELLEN[1]
[1]Dept of Microbiology, State University of New York Health Science Center at Brooklyn, 450 Clarkson Ave., Brooklyn, NY 11203, USA, [2]Moscow State University, Moscow 119899, Russia

Translation initiation usually involves ribosomal recruitment to the capped 5' end of mRNA by the eIF4E cap-binding subunit of eIF4F. Initiation on several viral RNAs is cap-independent and is mediated by an internal ribosomal entry site (IRES). We have characterized three mechanisms of IRES-mediated initiation.

Ribosomal attachment to the ~450nt. encephalomyocarditis virus IRES is mediated by the eIF4A and eIF4G subunits of eIF4F by binding the IRES independently of eIF4E. We mapped the overlapping IRES- and eIF4A-binding surfaces onto the 2.4Å structure of the central domain of eIF4G. Initiation on EMCV-like IRESs required additional IRES *trans*-acting factors that altered IRES conformation, enhancing binding of eIF4G/4A and thus initiation. Differences in ITAF expression patterns can account for cell type-specific differences in IRES function.

Initiation on the ~350nt. hepatitis C virus IRES occurred by a significantly different mechanism: the small ribosomal subunit bound the IRES directly in the absence of ATP and all initiation factors, including eIF4F. We used footprinting and mutagenesis of this and related IRESs to identify determinants of this specific interaction.

The ~180nt. intergenic IRES of the dicistronic Cricket paralysis virus genome also binds ribosomes in the absence of initiation factors; even more remarkably, this IRES has no requirement for initiation factors or initiator tRNA to begin translation. Instead, it induces ribosomes to enter the elongation phase of translation directly.

0945 Expressing alternative proteins by programmed frame shifting: tricks viruses use to confuse the ribosome

P. FARABAUGH
University of Maryland Baltimore County, Baltimore, USA

"Translational control" conventionally refers to any mechanism that changes the amount of completed product protein produced per unit amount of mRNA. Viruses also employ a different type of translational control, one regulating the structure of the encoded protein. Genes that use programmed translational frameshifting or programmed readthrough of termination codons produce two primary translation products. The first, corresponding one-to-one to the sequence of codons in the mRNA, results from following the canonical rules of translation. The second product is not explicitly predicted by the mRNA sequence because at a discrete point in the mRNA the rules of translation are changed to allow a shift in reading frame or bypassing of a termination codon. This type of event, called recoding, alters the rules of normal translational elongation through a variety of mechanisms. Perhaps a better term would be to "bend the rules" since mRNA sequences appear to enhance translational errors normally considered illegitimate. Some of those sequences interact with the ribosome to reduce its accuracy while others act indirectly on ribosome function. Studying how mRNA sequences interfere with translational fidelity may illuminate how ribosomes normally maintain accuracy.

1100 Control of plant virus mRNA translation by long distance rna:rna interactions

W. ALLEN MILLER, LIANG GUO, EDWARDS ALLEN and JENNIFER BARRY
Plant Pathology Dept., Iowa State University, Ames, IA 50011, USA

Barley yellow dwarf luteovirus (BYDV) provides an example of an mRNA that regulates translational processes via *cis*-acting sequences located far downstream of the site of action. Many plant viral RNAs lack the 5' cap and/or poly(A) tail that are essential for translation of most cellular mRNAs. Cap and poly(A) interact with translation factors to form a closed loop mRNA that greatly enhances ribosome recruitment. The uncapped, non-polyadenylated RNA genomes of BYDV and the large *Tombusviridae* family harbor translation elements (TEs) in their 3' untranslated regions (UTRs) that facilitate efficient initiation at the AUG nearest to the 5' end. We show that this interaction between mRNA termini depends on base-pairing (kissing) between stem-loops in each UTR. This kissing stem-loop is also required for viral RNA replication. The BYDV TE specifically binds the cap-binding initiation factor eIF4E, suggesting that ribosomes are recruited via canonical initiation factors. An independent long-distance kissing between a different stem-loop in the 3' UTR and one adjacent to the frameshift site facilitates the ribosomal frameshifting necessary for translation of the polymerase. This is unlike any other known frameshift signal. These results reveal remarkably specific, long-distance base-pairing interactions, and provide new possibilities by which viral RNAs may be translated.

1145 Adenovirus translational control – shunting ribosomes and remodelling of the cap-initiation complex

R. SCHNEIDER
New York University School of Medicine, USA

Adenovirus (Ad) inhibits translation of most cellular mRNAs during the late phase of virus infection by blocking the phosphorylation of eIF4E, while simultaneously carrying out the efficient translation of its own late family of mRNAs. We recently showed that the Ad 100k protein is responsible for inhibition of cellular protein synthesis. 100k protein competitively binds to translation factor eIF4G and displaces the eIF4E kinase known as Mnk1, which phosphorylates eIF4E and promotes cap-dependent translation of mRNAs. Ad late mRNAs possess a common 5' noncoding region (5'NCR), known as the tripartite leader, which confers the ability to translate exclusively by ribosome shunting when eIF4E is dephosphorylated. Ribosome shunting directed by the tripartite leader permits exclusive translation of viral late mRNAs after inhibition of cellular protein synthesis. We now show that the Ad late 100k protein is also a trans-acting, but specific, facilitator of translation by ribosome shunting on the tripartite leader. Tripartite leader reporter mRNAs were increased in shunting-specific translation about 10 fold by coexpression of a wild type 100k protein, compared to cellular eIF4F-dependent mRNAs that translate by ribosome scanning. Moreover, a temperature sensitive 100k protein mutant at restrictive temperature fails to promote shunting translation on the tripartite leader. The Ad 100k protein selectively stimulates ribosome shunting and not internal initiation of translation, as mRNAs containing the EMCV IRES were not enhanced in translation by coexpression of 100k protein. Sequence analysis of the 100k protein showed that it contains significant homology to the activating site of tyrosine phosphorylation found in Src kinase at position 416, as well as to other members of the Src family of tyrosine kinases. Immunoblot analysis of 100k protein with phosphotyrosine-specific antibodies showed it to be strongly phosphorylated during late Ad infection. Mutagenesis of the 100k protein Src-like sequence, to convert the tyrosine to phenylalanine (100k-phe), largely abolished tyrosine phosphorylation and the ability to promote translation by ribosome shunting on the tripartite leader. These and other data to be presented demonstrate that the Ad late 100k protein is a tyrosine kinase dependent, specific enhancer of translation by ribosome shunting on Ad late mRNAs.

1400 RNAs chasing their tails: a requirement for the initiation of poliovirus RNA synthesis

R. ANDINO
University of California, San Francisco, USA

The mechanisms and factors involved in the replication of positive-stranded RNA viruses are still unclear. Using poliovirus as a model, we show that a long-range interaction between ribonucleoprotein (RNP) complexes formed at the ends of the viral genome is necessary for RNA replication. Initiation of negative strand RNA synthesis at the 31-end requires a 31-poly(A) tail. Strikingly it also requires a cloverleaf-like RNA structure located at the other end of the genome. A ribonucleoprotein complex formed around the 51-cloverleaf RNA structure interacts with the poly(A) binding protein bound to the 31-end of the viral RNA thus linking the ends of the viral genome and effectively circularizing it. Formation of the 51-31 circular RNP complex is required for initiation of negative-strand RNA synthesis. RNA circularization may be a general replication mechanism for positive-stranded RNA viruses, as it may provide several advantages for viral replication, including the coordination of translation and RNA synthesis, the localization of the viral polymerase at the appropriate start site, and a control mechanism for the integrity of the viral genome.

1445 Structure/function analysis of a poliovirus *cis*-acting replication element (CRE) located in the middle of the genome

I. GOODFELLOW and D. EVANS
Division of Virology, Institute of Biological and Life Sciences, University of Glasgow, Church Street, Glasgow G11 5JR

The genome replication of single-stranded positive-sense RNA viruses involves the formation of a negative-strand template. In picornaviruses, the initiation of negative-strand synthesis is thought to involve sequences in the 3' non-coding region (NCR), the virus encode polymerase ($3D^{pol}$) and a virus-encoded VPg primer. We have recently identified a 61 nucleotide stem-loop structure within the region encoding the 2C protein which is absolutely required for replication (Goodfellow *et. al.* J.Virol 2000). Mutations which disrupt the RNA structure but leave the encoded protein unaltered prevent replication and are defective in negative-strand synthesis. The introduction of a second (unmodified) copy of the 2C loop restored the replication of a mutant in which the original CRE structure was disrupted. Paul and co-workers (Paul *et. al.* J. Virol 2000) have demonstrated that the poliovirus 2C CRE functions as template a for the uridylylation reaction of VPg (the formation of VPg-pUpU) that occurs during the initial stages of negative strand synthesis.

Using RNA structure probing and a cassette system (based on a poliovirus sub-genomic replicon) in which extensive changes to the loop can be made, we have identified the minimal sequence and structural requirements for the function of the CRE.

1500 Cleavage of translation initiation factor 4AI (eIF4AI) but not eIF4AII by foot-and-mouth disease virus 3C protease; determination of the eIF4AI cleavage site and the functional implications

W. LI[1], N. ROSS-SMITH[2], C.G. PROUD[1] and G.J. BELSHAM[2]
[1]School of Life Sciences, MSI/WTB Complex, University of Dundee, Dundee DD1 5EH, [2]BBSRC Institute for Animal Health, Pirbright, Woking, Surrey GU24 ONF

The eukaryotic translation initiation factor eIF4A (48 kDa) is the prototypic DEAD-box RNA helicase. It is required for cap-dependent and picornavirus internal ribosome entry site (IRES) dependent initiation of protein synthesis. Within cells infected by foot-and-mouth disease virus (FMDV), a picornavirus, the cleavage of eIF4A is observed and this requires the FMDV 3C protease. We now show FMDV 3C cleaves eIF4AI directly *in vitro* between residues E143 and V144. The closely related eIF4AII is not cleaved by this protease *in vitro* or within cells. Two amino acid substitutions within eIF4AI, near the cleavage site, conferred resistance to the protease. Alignment of the mammalian eIF4AI and yeast eIF4A sequences identified the position of the cleavage site within the 3D-structure of yeast eIF4A. The scissile bond is exposed and in a flexible portion of the molecule. Truncated versions of human eIF4AI lacking the N-terminal 100 residues, or more, failed to form a stable complex with eIF4G. The 3Cpro induced C-terminal cleavage product (33 kDa) of eIF4AI lacks the conserved ATPase A motif required for helicase activity and dissociates from the N-terminal cleavage product. Thus, the cleavage of eIF4AI by FMDV 3C is expected to inactivate each of its activities.

1515 The 5' UTR of Rhopalosiphum padi virus (RhPV) contains an internal ribosome entry site (IRES) which functions efficiently in mammalian, insect and plant translation systems

K.E. WOOLAWAY[1], G.J. BELSHAM[2], M.J. CARTER[1] and L.O. ROBERTS[1]
[1]School of Biomedical and Life Sciences, University of Surrey, Guildford, Surrey GU2 7XH and [2]Institute for Animal Health, Ash Road, Pirbright, Surrey GU24 0NF

Rhopalosiphum padi virus (RhPV) is one of several "picorna-like" viruses that infect insects; sequence analysis has revealed distinct differences between these agents and mammalian picornaviruses. RhPV has a single-stranded positive sense RNA genome of about 10kb, but unlike the genomes of picornaviruses, this contains two long open reading frames (ORFs). ORF1 encodes the virus non-structural proteins whilst the downstream ORF2 specifies the structural proteins. Both ORFs are preceded by long untranslated regions (UTRs). The intergenic UTR is known to contain an internal ribosome entry site (IRES) which directs a non-AUG-initiated translation of ORF2. We have examined the 5' UTR of RhPV for IRES activity by translating synthetic dicistronic mRNAs in a variety of systems. We report that the 5' UTR contains an element which directs internal initiation of protein synthesis from an AUG codon in mammalian and plant *in vitro* translation systems and in insect cells. In contrast, the encephalomyocarditis virus (EMCV) IRES only functions within the mammalian systems. The RhPV 5' IRES has features in common with picornavirus IRES elements in that no coding sequence is required for IRES function, but also with cellular IRES elements as deletion analysis indicates that this IRES element does not have sharply defined boundaries.

1600 Transport of intracellular enveloped vaccinia virus

G. RODGER[1], M. HOLLINSHEAD[2], H. VAN EIJL[2], M. LAW[2], R. HOLLINSHEAD[2], O. KRAUSS[2] and G.L. SMITH[1]
[1]The Wright-Fleming Institute, Imperial College School of Medicine, St Mary's Campus, Norfolk Place, London, [2]Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE

During vaccinia virus (VV) morphogenesis, intracellular mature virus (IMV) is wrapped by two membranes to form intracellular enveloped virus (IEV). Prior to release, IEV particles are then transported from the site of wrapping to the cell surface. Subsequent fusion of IEV at the plasma membrane results in the formation of cell associated enveloped virus (CEV) and the release of extracellular enveloped virus (EEV). Here we describe the mechanism for this movement as being mediated along microtubules.

To study IEV transport, we have constructed a recombinant VV that incorporates a GFP-fusion protein into the wrapping membranes and consequently, all virus particles derived from these membranes. This has allowed us to visualise the movement of these particles in real time. Results show that IEV particles move with a speed of ~ 60 ?m/min along discrete pathways and that this movement is inhibited by nocodazole which depolymerises microtubules.

1615 Coronavirus nucleoprotein localises both to the cytoplasm and nucleolus

T. WURM[1], H. CHEN[1], P. BRITTON[2], T. HODGSON[1], G. BROOKS[1] and J. HISCOX[1]
[1]School of Animal and Microbial Sciences, University of Reading, [2]Division of Molecular Biology, Institute for Animal Health, Compton

The coronavirus nucleoprotein (N protein) is the most abundant virus-derived protein produced throughout infection. Several functions have been postulated for the N protein. Primarily, it complexes with the coronavirus genomic RNA to form a ribonucleocapsid structure, and has also been postulated to have a role in replication of the genomic RNA.

Using confocal microscopy, we investigated the intracellular localisation of several coronavirus N proteins in both the absence and presence of other viral proteins, and found that each N protein localised to both the cytoplasmic and nucleolar compartments suggesting that this phenomenon is common to the coronaviruses and a general feature of all the *Nidovirales*. N protein was used to target a reporter protein to the nucleolus, and we identified a nucleolar localisation signal within the N protein.

As the nucleolus is the site of ribosome synthesis, we postulated that the coronavirus N protein might localise to the nucleolus as part of a strategy to control both viral and host cell translation. Alternatively, proteins that associate with the nucleolus have been implicated in the control of the host cell cycle, and the coronavirus N protein therefore might have a similar role.

1630 Effect of HCV core protein on HCV IRES-mediated translation

M. SCOTT, J. WOOD and A.H. PATEL
MRC Virology Unit, Church Street, Glasgow G11 5JR

The HCV IRES has been shown to be translationally active in mammalian cells but not in insect cells. We examined the possible role of the viral core protein in relieving this apparent translational block in insect cells. Recombinant baculoviruses (rbacs) expressing HCV core (rbac-C), 5' non-coding region (NCR) linked to core (rbac-5C), or 5C fused in-frame with the bacterial CAT gene (rbac-5CC) were generated. As expected, we were able to detect appropriate transcripts, but not their protein products in Sf cells infected with rbac-5C, or rbac-5CC which both possess the HCV IRES within the 5'NCR. In contrast, rbac-C and rbac-CC, which lack the 5' NCR, expressed HCV core in substantial amounts and gave high level CAT activity, respectively. Co-infection of rbac-C and rbac-5CC resulted in a 4.2-fold elevation of CAT activity compared to the rbac-5CC single infection, suggesting that core protein itself can alter IRES activity. The ability of core to alter IRES activity in an Sf cell assay has been reproduced in human hepatocytes (HuH-7). Expression of core in HuH-7 cells specifically upregulated CAT activity of co-transfected 5CC constructs by approximately 5-fold. We are currently examining whether core protein or its encoding RNA mediates the observed effect.

1645 An investigation into RNA secondary structural elements critical to retroviral replication

T.P. MONIE, J.S. GREATOREX and A.M.L. LEVER
Dept of Medicine, University of Cambridge, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QQ

RNA structures have been attributed critical roles in both cellular and viral replication systems. Within the Retroviridae RNA structural motifs are associated with important functional roles, including, genomic RNA encapsidation, the nuclear export of transcripts and dimerisation of the viral genome.

We are studying the nature of one such element, the Dimer Linkage Site (DLS), in a selection of diverse retroviruses including, Human T-Cell Lymphotropic Virus Type 1 (HTLV-1) and the sheep pathogen Maedi Visna Virus (MVV). This will address the structural variation between different DLS's and also the functional role of dimerisation within the viral life cycle.

Previous work had isolated a 32 nucleotide viral sequence responsible for dimerisation of HTLV-1. RNA oligonucleotide fine mapping of this region isolated a palindromic sequence, predicted to form a putative stem-loop, which was critical for dimerisation. Secondary structure mapping of this region will confirm the nature of the interactions involved.

The host range, pathogenesis and cell tropism of MVV differs from HTLV-1 and the DLS of the virus has not previously been investigated. In vitro dimerisation assays and Northern blotting of infected cells and virions shall determine whether this virus does dimerise and also locate the DLS within the genome.

1700 Role of the respiratory syncytial virus (RSV) G protein in immunopathology

C. MAHER[1,2], P. ERTL[2], C. RING[2], E. BLAIR[2], T. HUSSELL[1] and P. OPENSHAW[1]
[1]Respiratory Medicine Dept, Imperial College at St Mary's, Norfolk Place, London W2 1PG, [2]Virology Unit, GlaxoWellcome R&D, Gunnells Wood Road, Stevenage SG1 2NY

Respiratory syncytial virus (RSV) is the most important lower respiratory tract pathogen of infancy; current therapies are supportive or expensive and toxic and no effective vaccine is yet available. The G (putative attachment) protein of RSV is highly glycosylated with both *N* and *O* linked sugars. The biological role of these sugars is unknown. We therefore used reverse genetics to produce glycosylation mutants of RSV.

We used site-directed mutagenesis to disrupt potential N-linked glycosylation sites within the G protein cDNA. Resulting recombinant RSV was rescued and sequenced in entirety. The phenotype of these recombinant viruses was studied *in vitro* and *in vivo*.

Growth in cell culture was attenuated and small plaque morphology was observed. Mutant viruses were infectious in BALB/c mice. We will evaluate these viruses further and investigate them as vaccine candidates.

1715 Molecular epidemiology of rabies virus isolates from France

J. SMITH[1], N. JOHNSON[1], L. MCELHINNEY[1], J. BARRAT[2], F. CLIQUET[2] and A.R. FOOKS[1]
[1]Rabies Research and Diagnostic Group, Dept of Virology, Veterinary Laboratories Agency (Weybridge), New Haw, Addlestone, Surrey, KT15 3NB, [2]AFSSA, Nancy, Laboratoire d'Etudes sur la Rage et la Pathologie des Animaux Sauvages, BP 9, 54220 Malzeville, France

Classical rabies viruses are defined as genotype 1 of the Lyssavirus genus within the Rhabdoviridae family, and remain endemic in many areas of Central and Eastern Europe. This study was designed to investigate sequence variation within Eastern France where rabies was endemic before the oral vaccine baiting strategy was implemented. Viruses (n=251) were isolated from a range of infected host species including foxes, cows and sheep, in the Northeast of France between 1988-89. In this investigation we have analysed the N-terminal 405 base-pairs of the nucleoprotein (N) gene. All of the 251 isolates were shown to be genotype 1. Phylogenetic analysis of this data set has revealed genetically distinct groups of viruses within the genotype, related by host species with a geographic distribution. Genetic sequence analyses of these viral isolates may provide important information on the epidemiology of this virus.

FRIDAY 30 MARCH 2001

0900 HSV pathogenesis: a disruptive guest's interaction with a model host

DAVID A. LEIB
Depts of Ophthalmology and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA

Herpes simplex virus induces the rapid shutoff of macromolecular synthesis in infected cells. The factor responsible for this shutoff is the product of the UL41 gene known as the virion host shutoff (*vhs*) protein. The *vhs* protein acts through the selective destabilization of mRNA and is conserved among all of the neurotropic herpesviruses. Surprisingly, this gross alteration of host cellular metabolism is dispensable for viral replication in cell culture. HSV recombinants lacking *vhs* however are profoundly attenuated in animal models, in terms of their abilities to replicate in all tissues, cause disease, as well as to establish and reactivate from viral latency. Despite this significant attenuation, such recombinants remain highly immunogenic and serve as highly effective prophylactic and therapeutic vaccines, with a stimulation of a strong delayed-type hypersensitivity response. These data suggest a role for *vhs* in disrupting aspects of the host immune response. Another HSV protein, ICP34.5, precludes the shut-off of protein synthesis in infected cells which is mediated by the interferon (IFN)-inducible double-stranded RNA-dependent protein kinase or PKR. ICP34.5 is also a potent neurovirulence determinant for the virus. A virus that has been profoundly attenuated by deletion of ICP34.5 exhibits wild-type replication and virulence in a host from which the PKR gene has been deleted, and restoration of virulence is specific to ICP34.5 and PKR. This specific phenotypic restoration defines a key molecular target of ICP34.5 *in vivo* and provides genetic criteria for determining mechanisms of microbial virulence *in vivo*.

0945 Analysis of the aphthovirus 2A.2B polyprotein 'Cleavage" mechanism indicates not a proteolytic reaction, but a novel translational effect: a putative ribosomal 'Skip'

M.D. RYAN
University of St Andrews

The strategy of encoding proteins in the form of a polyprotein appears to be ubiquitous amongst positive-stranded RNA viruses. 'Processing' of these polyproteins is, perhaps, synonymous with post-translational proteolytic processing cleavage of the polyprotein at specific sites by either virus-encoded or host-cell proteinases. The primary, co-translational, 2A/2B cleavage of the aphtho- and cardioviruses is mediated by 2A 'cleaving' at it's own C-terminus. In aphthoviruses this region is very short (~18aa), and, together with the N-terminal residue of protein 2B (a conserved proline residue) represents an autonomous 'self-cleaving' element.

Characterisation of the 'cleavage' has lead us to propose that it is brought about by a novel manipulation of translation, rather than by proteolysis. Specifically that this sequence modifies the activity of the ribosome to promote hydrolysis of a peptidyl-tRNA ester linkage releasing the polypeptide from the translational complex such that synthesis of a discrete downstream translation product may ensue: 'skipping' from one codon to the next without the formation of a peptide bond.

'2A-like' sequences have been identified in a number of cellular proteins, insect virus polyproteins, rotaviruses type C gene 6 and repeated sequences in *Trypanosoma* spp. The possible roles of active '2A-like' sequences will be discussed.

1100 Interaction of adenoviral RNAs with PKR

M.B. MATHEWS, I. FIERRO-MONTI and L.M. PARKER
Dept of Biochemistry and Molecular Biology, New Jersey Medical School, UMDNJ, Newark, NJ 07103-2714

The structured adenoviral RNA, VA RNA_I, is important for viral replication. Approximately 80% of adenoviral serotypes also encode a second structured RNA species, VA RNA_{II}. A mutant virus lacking VA RNA_I replicates poorly as a result of the activation of the dsRNA-activated cellular kinase PKR. This phenotype is exacerbated in a double mutant lacking both VA RNA_I and VA RNA_{II}, although a virus lacking VA RNA_{II} alone replicates with nearly wild-type efficiency. These findings are consistent with overlapping but distinct functions for these two RNAs. It is well established that VA RNA_I, but not VA RNA_{II}, strongly inhibits PKR thereby preventing it from phosphorylating translation initiation factor eIF2. The cellular protein NF90 preferentially interacts with VA RNA_{II} and can directly interact with PKR. Therefore, we considered the possibility that NF90 might modulate PKR activity.

We find that NF90 is a substrate for, and regulator of, PKR. NF90 contains two dsRNA-binding motifs (dsRBMs) and binds to dsRNA (and, to a lesser extent, ssRNA) through these motifs. NF90 inhibits PKR kinase activity *in vitro*: this inhibitory activity maps to the dsRBMs in the protein's C-

terminus, and can be overcome by high concentrations of dsRNA, implying competitive binding to dsRNA. In addition, the N-terminus of NF90 interacts with PKR in an RNA-independent fashion. In yeast growth assays, NF90 exacerbated the growth phenotypes caused by PKR. This effect correlates with eIF2 phospho-rylation. NF90 has no effect on yeast growth when expressed alone or in the presence of the catalytically-defective K296R mutant of PKR. Interestingly, a similar effect of NF90 was seen on HRI, another eIF2 kinase, when expressed in yeast. These data suggest that the stimulatory effect of NF90 on PKR in yeast may work through interactions with eIF2, perhaps by forming a ternary complex with PKR. In support of this model, NF90 interacts with eIF2 in co-immunoprecipitation experiments.

1145 Sendai virus replication and its interaction with the host cell

D. KOLAKOFSKY
University of Geneva Medical School, Switzerland

Abstract not received

Abstracts Book – Poster Abstracts - Late submissions

Cells & Cell Surfaces Group

CCS 05 Bacterial cytoskeleton: cell shape determination in *Bacillus subtilis*

Rut CARBALLIDO –LÓPEZ
Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE

A fundamental question in cell biology is how cell shape is determined. In the absence of an overt cytoskeleton, the external cell wall of bacteria has traditionally been assumed to be the primary determinant of cell shape. In the Gram-positive bacterium *Bacillus subtilis*, the *mbl* gene was shown to be required for cell morphogenesis, being important in maintaining the linearity of the longitudinal axis of the cell. Mbl is present in different species of bacteria with non-spherical and therefore "actively" determined cell shape. Despite a weak sequence homology, structural similarity to eukaryotic actins had been predicted. Sub-cellular localization of Mbl protein by GFP fusions and by immunofluorescence microscopy revealed helical filamentous structures, which lie close to the cell surface and run the length of the cell. A combination of cross-linking and sedimentation studies demonstrated Mbl self-assembly in both *B. subtilis* cell extracts and with purified recombinant protein. Filamentous ordered structures and fibrous aggregates were observed by electron microscopy. Mbl self-assembly was also shown to be reversible, a common property of dynamic cytoskeletal proteins. These results suggest that Mbl polymers probably form the helical structures observed within the cell and that they play a direct role in morphogenesis. They support the notion that Mbl is a bacterial cytoskeletal element and they further extend the functional homology between Mbl and eukaryotic actins, strengthening the suggestion that they are homologous having evolved from a common ancestor.

Fermentation & Bioprocessing and Physiology, Biochemistry & Molecular Genetics Groups

FbPbmg 17 Bacterial biodegrading of arsenobetaine in monoseptic culture

A.W. RITCHIE, N. MOLENAT, J.S EDMONDS, C.F. HARRINGTON and R.O. JENKINS
Dept of Biological Sciences, De Montfort University, The Gateway, Leicester LE1 9BH

Arsenobetaine [$(CH_3)_3As^+CH_2COO^-$; AB] is an arsenic-containing non-toxic organic compound that is considered to be ubiquitous in the marine environment and is generally regarded as a key compound in environmental arsenic cycling. Whereas biodegradation of AB under aerobic conditions by undefined mixed-cultures and by defined bacterial consortia has been reported previously, there are no reports of AB biodegradation by monoseptic cultures of bacteria.

We present evidence for AB biodegradation, involving cleavage of the C-As bond, by isolates from the marine environment when grown in monoseptic culture. ^{1}H NMR and HPLC-AAS were used to screen for AB biodegrading capability in aerobic enrichment cultures involving inocula from the marine environment. Trimethylarsine oxide (TMAO), dimethylarsinic acid (DMAA) and methylarsonic acid (MAA) - potential breakdown products of AB - were detected after microbial enrichment of mussel extract on mineral salts medium containing glycinebetaine (the N containing analogue of AB) as main carbon and nitrogen source. Isolates from positive enrichment cultures were tested for AB biodegradation in monoseptic culture using HPLC-hydride generation-atomic fluorescence spectrometry. DMAA was detected after 21-days incubation in monseptic cultures of *Brevundimonas versicularis*, *Aeromonas salmonicida*, *Flavobacterium* sp and *Pseudomonas* sp. These data suggest that the isolates degrade AB to DMAA via a route not involving TMAO, possibly through demethylation of AB prior to C-As bond cleavage, i.e. involving dimethylarsinoyl acetic acid as an intermediate. Detection of TMAO in the undefined mixed cultures and its apparent absence in monoseptic cultures suggests that two pathways of AB biodegradation exist, located in different bacterial species.

FbPbmg 18 A microbial basis for biomethylation of antimony in anaerobic environments

LOUISE M. SMITH and RICHARD O. JENKINS
Dept of Biological Sciences, De Montfort University, The Gateway Leicester LE1 9BH

Antimony (Sb) is found naturally in the environment in trace quantities, but widespread industrial utilisation has redistributed this element so that it intermingles with biological food chains at high concentrations. Whereas the biotransformations of arsenic - a Group 15 cogener of antimony - have been intensively studies, those governing the mobility and toxicity of antimony are poorly understood. Methylantimony species have been found in various natural and man-made environments, while certain aerobic fungi and mixed cultures of anaerobic bacteria are known to biomethylate inorganic antimony.

We present evidence for antimony biomethylation by monoseptic cultures of clostridia. Gas chromatography - mass spectrometry (GC-MS) was used to screen for antimony biovolatilisation capability in various soils by anaerobic enrichment culture. Trimethylantimony (TMA) was found in the headspace of cultures designed to promote growth of clostridia. GC-MS profiles of headspace gases of mixed cultures able to generate TMA was used to select *Clostridium* spp for testing for antimony biomethylation capability, by hydride generation - gas chromatography - atomic absorption spectrophotometry. Methylantimony species were detected in the culture medium of monoseptic cultures of *C. acetobutylicum*, *C. butyricum* and *C.cochlearium*. Two clostridial isolates from soil enrichment culture were also shown to biomethytlate inorganic antimony in monoseptic culture. Antimony biomethylation by clostridia, which are known to participate in the multistage process of methanogenesis, may account for the presence of volatile antimony in biogas from landfill sites. The relative quantities of mono-, di- and trimethylantimony species produced illuminates the mechanism of antimony biomethylation in these bacteria.

FbPbmg 19 Enzyme-coated micro-crystals: A 1-step method for high activity biocatalyst preparation

MICHAELA KREINER[1], BARRY D. MOORE[2] and MARIE CLAIRE PARKER[1]
[1]Dept of Chemistry, University of Glasgow, University Avenue, Glasgow G12 8QQ,
[2]Dept of Pure and Applied Chemistry, University of Strathclyde, 295 Cathedral Street,
Glasgow G1 1XL

Over the past decade biocatalysis has become a routine tool in organic synthesis and a number of industrial processes now utilise enzyme-catalysed reactions in low-water organic media.
Here we describe a novel method for isolating a protein from aqueous solution by simultaneous dehydration and co-precipitation. The resultant enzyme preparation consists of micron-sized crystals of an inert component, such as a sugar or salt that is coated with protein molecules. Preparation of the enzyme-coated micro-crystals is fast, inexpensive and requires only standard laboratory equipment. The protein-coated micro-crystals are easy to dispense from organic solvent and show high stability and dramatically enhanced activity in organic solvents. Alternatively, re-dissolution in aqueous solution is possible.

Here we demonstrate this precipitation method using the protease subtilisin Carlsberg and lipases of differing origin and purity. Remarkably, the catalytic rate of subtilisin Carlsberg was 10^3 faster than typically found using lyophilised preparations. The catalytic activity of the lipases could be improved up to 200-fold, depending on the lipase.

Together with this, exploration of the morphology and presentation of the protein nanoclusters at the crystal surface have been performed using a combination of electron microscopy (EM) and atomic force microscopy (AFM) to directly image the protein-bound layer.

FbPbmg 20 Use of a biocatalytic scrubber to detoxify acrylonitrile from waste gas streams

P.C.J. ROACH[1], P. WILLIAMS[1], D.K. RAMSDEN[1], K.C. SYMES[2] and J. HUGHES[2]
[1]University of Huddersfield, [2]Ciba Specialty Chemicals

Acrylonitrile contaminated air is a common by-product of chemical industries which utilise acrylonitrile (e.g. BP; BASF). Presently, the removal of these toxic vapours is difficult because of the low solubility of acrylonitrile, only 7% w/v (approximately 1.3M) in water. Furthermore, water saturated with acrylonitrile has little industrial value and remains as toxic as the contaminated air, still posing a disposal problem. One alternative is to hydrolyse the acrylonitrile to ammonium acrylate using the very low Km nitrilase

contained within the micro-organism *Rhodococcus ruber*. Ammonium acrylate is a non-toxic compound and is also of commercial value.

Using this microorganism acrylonitrile scrubbing has been carried out in a conventional scrubber with a suspension of the micro-organism in the liquid phase (scrubber fluid). The microorganism has also been immobilised on a particle support within a column so that the rate of contact between the immobilised cells and the acrylonitrile vapour is increased. . Immobilised cells are a more viable option than use of free cells within the system, as biocatalyst replacement is required less frequently.

Acrylonitrile vapour detoxification was achieved using an immobilised biocatalyst. The porous nature of the immobilising matrix used (inert silicon) allowed dense packing of micro-organisms so that high removal efficiencies were achieved. The advantage of using immobilised nitrilase cells is that there is no start-up period, the biocatalyst is more stable to flow interruptions and the resultant ammonium acrylate solution needs no further treatment prior to use, sale or disposal. The system is readily adaptable for use with other volatile waste gases, or indeed liquid waste.

FbPbmg 21 Development of a microbial process for enantioselective biotransformations using biocatalysts

S. HUSSAIN[1], P. WILLIAMS[1], D.K. RAMSDEN[1], K.C. SYMES[2] and J. HUGHES[2]
[1]University of Huddersfield, [2]Ciba Specialty Chemicals

The University of Huddersfield (Biotechnology Centre) and Ciba Specialty Chemicals have been collaborative partners in a programme to develop and exploit biocatalytic technology for over a decade. A result of this collaboration has been the study of commercially viable bioconversion processes such as the enantioselective hydrolysis of racemic α-substituted aromatic nitriles to the R-amide and the S-acid with an enantiomeric excess (e.e) of >98%.

Microbial strains were initially isolated using enrichment culture techniques. These techniques specifically selected for microorganisms which were capable of both resisting the cytotoxic effects of aromatic/aliphatic nitriles and amides and catalysing their degradation. To degrade such compounds, the microorganisms required enzymes such as nitrilase, nitrile hydratase and amidase.

The *Rhodococcus wratslaviensis* strain utilised in this work has been shown to have a time independent stereoselective amidase which is capable of selectively converting racemic phenylglycineamide to S-phenylglycine. The concentration of enantiomerically pure product can exceed its solubility product and thus readily precipitates out of solution for simple recovery. The unconverted R-phenylglycineamide is not a substrate for the microbial strain used and remains in solution.

Systematics & Evolution Group

SE 09 Testing the Complexity Hypothesis

RAVI JAIN, MARIA RIVERA and JAMES LAKE
Molecular Biology Institute, and MCD Biology, University of California, Los Angeles, CA 90095, USA

The Complexity Hypothesis was proposed to explain why some groups of genes are subject to frequent horizontal transfer, the operational genes, while others, the informational genes, are not. The Complexity Hypothesis proposes that the number of protein, or RNA, contacts made by a gene product differentiates these two superclasses of genes. Proteins of the operational class, on average, make fewer contact with other proteins, than informational proteins. The difference in protein/protein interactions makes it more likely that an operational gene, whose product interacts with no, or very few, other proteins, will be retained in the genome of a prokaryote that has horizontally acquired it because the operational protein can carry out its function nearly independently of the host proteins. On the other hand, an informational protein may require many protein interactions for functioning in a complex. For a prokaryote only distantly related to the source of a horizontally acquired gene, those proteins having the fewest interactions are the most likely to function in a new cellular environment.

To test whether the number of protein (or RNA) contacts affects the frequency of successful horizontal transfer, we have determined the number of protein/protein interactions for all *E. coli* proteins that are available in the Database of Interacting Proteins. We are also searching for other factors that may help explain the observed differences in horizontal transfer. First, we are rigorously determining orthologs of the *E. coli* genes (in collaboration with M. Riley) to build phylogenetic trees and to measure directly the numbers of horizontal transfers using the Steel algorithm. Second, we are calculating mono- and di-nucleotide frequencies for ORFs,in order to correlate nucleotide usage with lateral transferability. We are also correlating codon usage and horizontal transfer. The study of complexity using these parameters promises to provide insight into the factors affecting horizontal transfer, including the complexity hypothesis. Our latest results will be presented.

SE 10 Evolution of genome rearrangements In ciliates

LAURA A. KATZ[1,2] and JENNIFER L. RILEY[1]
[1]Dept of Biological Sciences, Smith College, Northampton, MA, USA, [2]Graduate program in Organismic and Evolutionary Biology, University of Massachusetts, MA, USA

Ciliates are defined by the presence of dimorphic nuclei in every cell; both the functional macronucleus (MAC) and the transcriptionally inactive germline micronucleus (MIC) develop from a zygotic nucleus following conjugation. As the MAC develops, germline chromosomes are processed by fragmentation, excision of internal sequences and amplification of the remaining chromosomes. Extensive processing of chromosomes to generate a MAC with gene-sized fragments (defined here as average size less than five kilobases (kb)) has been well documented from only one class of ciliates. We demonstrate the broad distribution of extensive fragmentation among members of the class Phyllopharyngea and in the genera *Metopus* and *Nyctotherus*. We analyze small subunit rRNA genealogies from these taxa to show independent origins (or multiple losses) of extensive fragmentation among ciliate lineages. These data indicate that the process of extensive fragmentation is more widespread within ciliates than had previously been thought and provides a new framework for interpreting the evolution of chromosomal rearrangements within ciliates.

SE 11 Biogeography and level of endemism in ciliate populations from coastal environments

OONA L.O. SNOEYENBOS-WEST[1], TOVAH SALCEDO[1], GEORGE B. McMANUS[2] and LAURA A. KATZ[1,3]
[1]Dept of Biological Sciences, Smith College, Northampton, MA USA, [2]Dept of Marine Sciences, University of Connecticut, CT USA, [3]Graduate program in Organismic and Evolutionary Biology, University of Massachusetts, MA USA

In 1934 Baas-Becking transformed microbial ecology with the now famous quote that "Everything is everywhere, the environment selects." This axiom has since found wide acceptance, despite the paucity of data on microbial biogeography. Such data is needed to test this idea since it has vast implications for our understanding of microbial biodiversity and evolution. We are using molecular markers to assess interspecific and intraspecific variation in natural populations of putatively cosmopolitan oligotrich and choreotrich ciliate species across space and time. In order to elucidate relationships among these taxa, we sequenced small subunit ribosomal RNA and α-tubulin genes from four choreotrichs and three oligotrichs. Phylogenetic analyses support the monophyly of the choreotrichs while the oligotrichs appear polyphyletic. To explore the level of genetic variation among individuals in geographically and temporally isolated populations, we characterized DNA polymorphisms in the internally transcribed spacer region 1 (ITS-1) and region 2 (ITS-2). Our preliminary data indicate that at least for some ciliate populations, biogeographic separation does exist, and that ciliate diversification is an ongoing process. We are pursuing other molecular markers and predict that future environmental genomic studies will have a large impact on our understanding of protist biogeography.