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MONDAY 10 SEPTEMBER 2001

Systematics

0910 *Mycobacteria in the 21st century*

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Tuberculosis and leprosy ravaged Europe for centuries, but incidence has fallen dramatically due to a number of social, biological and public health interventions. Scientifically, the 19th century saw the identification of the causative organisms, while the 20th century brought a vaccine, antibiotics, and the genome sequence. However, as we move into the 21st century, the incidence of TB is rising in many low-income countries, driven by the HIV pandemic. Multidrug-resistant strains of *Mycobacterium tuberculosis* have emerged, and large trials have shown BCG to be ineffective in the parts of the world that need it most. In addition, bovine tuberculosis is a major problem in the UK, but vaccination is not possible because it compromises skin testing. There is therefore a pressing need for novel antibiotics and a better vaccine. Because of their medical and veterinary importance, the mycobacteria are now the most deeply sequenced group of organisms. The combination of comparative genomic analysis, genetic tools such as targeted gene inactivation, and functional genomics technologies provide an opportunity for a quantum leap in our understanding of the biology of these pathogens.

0950 *Systematics of mycobacteria*

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The rekindling of interest in mycobacterial systematics started with the Runyon revolution but continued to reverberate with the introduction and application of chemotaxonomy, molecular systematics and numerical phenetic taxonomy. The impact of the methods, both individually and collectively, in shaping the classification of mycobacteria and other mycolic acid containing actinomycetes will be covered. These organisms belong to the suborder *Corynebacterineae* which encompasses nine genera which form a distinct clade in the 16S rDNA tree. Over half of the 92 validly described species of *Mycobacterium* contain organisms that are considered to be pathogenic for animals and man. The importance of this classification in the recognition, characterisation and identification of putatively novel and established species of *Mycobacterium* will be highlighted. In addition, the integrated use of genotypic and phenotypic methods in unravelling the taxonomy of poorly studied, albeit clinically significant groups of mycobacteria will be illustrated. The prospects and implications of further improvements in the classification of mycobacteria will be considered.

1100 *Comparative mycobacterial genomics*

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Mycobacterium tuberculosis, the scourge of humanity, is one of the most successful and scientifically challenging pathogens of all time. The complete 4.41 Mb genome sequence is available for H37Rv, the paradigm strain for the slow-growing *M. tuberculosis*

complex. Bioinformatic analysis led to the identification of ~4,000 genes in the genome sequence, and provided fresh insight into the biochemistry, physiology, genetics and immunology of this much-feared bacterium. The information and knowledge thus obtained is now catalyzing the conception of new prophylactic and therapeutic interventions against tuberculosis, and enhancing our understanding of the biology of the tubercle bacilli. The abundance of genes predicted to be involved in lipid and polyketide metabolism suggests that lipolysis is likely to be the major metabolic source of energy and carbon. Prominent amongst the "orphan" genes were two large families encoding novel, glycine-rich proteins of repetitive structure, the PE and PPE proteins. Over 8% of the genome is devoted to the production of these curious proteins of unknown function and this implies that they must play an important biological role.

Comparative genomics of other members of the *M. tuberculosis* complex, using BAC-arrays, has uncovered a series of variable loci that may be responsible for a variety of phenotypic differences including host range and virulence. In parallel, the 3.3 Mb genome sequence of the related leprosy bacillus, *Mycobacterium leprae*, has been determined. Comparisons of the two sequences are mutually enriching and have revealed numerous pseudogenes and extensive genetic decay in *M. leprae*. Genome downsizing may account for the exceptionally slow growth of this unculturable pathogen while the evolution of *M. leprae* has naturally defined the minimal gene-set for mycobacteria.

1140 *Mycobacterial cell wall*

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Chemical definition of the cell wall of *Mycobacterium tuberculosis*, notably the mycolic acids, arabinogalactan, and peptidoglycan, was undertaken in the 1960s by such as D.E. Minnikin and several French workers under E. Lederer. With the advent of superior analytical tools in the 1980s, we (with M. McNeil, G.S. Besra, M. Daffé) readdressed the topic, such that nowadays we have a good comprehension of the primary structure of the huge covalent complex, the mycolylarabinogalactan peptidoglycan that comprises the mycobacterial cell wall core. More recently, we have addressed the biosynthesis of this complex, demonstrating that it is initiated by the synthesis of a linker disaccharide on a polyprenyl-P followed by the sequential addition of single Galf units donated by UDP-Galf and catalyzed by the Rv3808c gene product. The donor of the Araf units has been recognized as C₅₀-P-Araf and the enzymology and genetics of arabinan synthesis is now being intensely investigated. Little is also known about the terminal steps in cell wall core assembly, i.e., the addition of mycolic acids and the ligation of the complex to peptidoglycan. This work is important in the context of efforts to develop new drug treatments for tuberculosis.

Epidemiology

1400 Tuberculosis epidemiology and environmental influences

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The "environment" has a profound influence on tuberculosis. There is much evidence that infection transmission is associated with crowded, poorly-ventilated conditions as obtain in poor urban areas, and sunlight exposure itself reduces survival of tubercle bacilli and hence reduces transmission. Such influences may explain at least in part some well known dramatic differences between tuberculosis in different times and settings. The decline in tuberculosis in the West which began in the 19th century, prior to medical interventions, was associated with improved living conditions, and the fact that tuberculosis was worse in urban populations of the 19th century Europe than anywhere in developing countries today may be linked to the greater ventilation and sunlight exposure in warmer climates.

There is also evidence that the microbiological environment exerts an important influence upon tuberculosis. Many species of "environmental" mycobacteria live in soil and water habitats throughout the world, in particular in low-latitude tropical or subtropical areas. Epidemiological studies in human populations as well as animal studies show convincingly that exposure to and sensitisation by certain of these mycobacteria can impart varying degrees of protection against tubercle disease. This may add to the explanations for the predilection of tuberculosis for urban environments, given that environmental mycobacterial exposure is likely to be more intense in rural than in urban areas. Finally, exposure to environmental mycobacteria provides an explanation for the observed variations in protection by BCG vaccination between human populations – the argument being that exposure to certain environmental mycobacteria can provide as much protection as can BCG. Being unable to improve upon this naturally acquired protection BCG appears to be useless in such environments.

1440 Bovine tuberculosis: current epidemiological issues

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After all cattle herds in Great Britain became attested for bovine TB in 1960, the great majority of new cattle cases have been in the west of the country. This has focussed attention on possible wildlife reservoirs of infection, and during the last 25 years the finger has been pointed particularly at the badger. However, the extent of this species' involvement in transmitting *Mycobacterium bovis* infection to cattle is still the subject of both debate and extensive research effort directed towards elucidating the role of this and other possible wildlife reservoirs in TB transmission. Furthermore, cattle-to-cattle transmission may still be an important element not only in the spread of disease from herd to herd but also in determining the extent of infection *within* a herd. The single comparative intradermal tuberculin test was the diagnostic basis for the original eradication programme and remains, with little modification, the principal means of detecting herd infection in the present control programme. However, no diagnostic test is perfect and the possibilities of reservoirs of undetected infected, infectious, or intermittently excreting cattle have been suggested. There is currently a wide ranging research programme not only investigating these issues but also directed towards improving TB control through such means as vaccine and

diagnostic test development, and application of molecular typing methods. This paper considers current epidemiological questions in bovine TB in Great Britain, drawing on current research in the hunt for answers.

1550 The role of *Mycobacterium tuberculosis* Beijing genotype strains in the world wide tuberculosis epidemic

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In areas with a high incidence of tuberculosis, like Central Africa and Asia, *Mycobacterium tuberculosis* isolates show a much lower degree of genetic polymorphism than in low incidence areas. The most pronounced example of evolutionary conservation among *M. tuberculosis* isolates was found in China, where more than 80 percent of the isolates exhibited at least 75% similarity among the multi-banded IS6110 RFLP patterns, and identical spoligopatterns. Latter group of strains, which later on also appeared to be present in high frequencies in other parts of Asia, was designated the 'Beijing' genotype. In a study in Vietnam in 1999, the occurrence of this genotype was found significantly associated with low age of patients and, hence, with active transmission (emergence) of tuberculosis. Moreover, in Vietnam, Indonesia, and e.g. former USSR republics strong correlations were reported between the Beijing genotype strains and (multidrug) resistance. It seems plausible that the successful worldwide spread of Beijing genotype strains can be explained by particular selective advantages. So far, several observations indicated that both an enhanced capacity to gain resistance and an altered interaction with the host immune defence system may play a role in this respect. In Vietnam, relapses of tuberculosis after curative treatment were found significantly associated with Beijing genotype strains. In experiments in Macaques monkeys and BALB/c mice, immune responses were significantly lower after infections with Beijing genotype strains as compared to the ones with other *M. tuberculosis* strains. Moreover, the altered induction of immune responses against Beijing genotype strain infections was recently confirmed in practice; patients in Indonesia infected with these strains twice as often developed fibrile responses during treatment.

1630 Nonreplicating persistence of *Mycobacterium tuberculosis*

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A major impediment to the control and eradication of tuberculosis is the ability of *Mycobacterium tuberculosis* to persist in host tissues for long periods of time without increasing in numbers, but still retaining the ability to resume replication when conditions become favorable. Months or years may elapse between initial infection of a human host and appearance of disease. Similarly, tubercle bacilli may replicate vigorously in experimental animals for several weeks after inoculation, but replication may cease when cell mediated immunity is activated, and the number of bacilli in the tissues then remains constant for

months. Although great progress has been made in explicating the complex mechanisms of the host's immune response, less is known about the physiologic responses of the bacilli themselves to the modified host environment. Oxygen depletion is a common factor in inflammatory and necrotic tissues. *M. tuberculosis* can replicate in vitro only when the medium is at least 10% saturated with oxygen (with respect to air). If the oxygen is abruptly depleted, the bacilli will die. If the oxygen is depleted slowly, tubercle bacilli undergo a series of adaptive microaerophilic changes that permit them to survive hypoxically in a nonreplicating state. Among the adaptive changes that facilitate this state of nonreplicating persistence are selective termination of energy dependent macromolecular syntheses, induction of alternative energy pathways, and stabilization of essential proteins to minimize the need for their ongoing synthesis. A broader understanding of mechanisms involved in the hypoxic shiftdown of *M. tuberculosis* to nonreplicating persistence may permit development of antimicrobial agents which can prevent that persistence.

TUESDAY 11 SEPTEMBER 2001

Pathogenesis

0900 *Mycobacterium tuberculosis* virulence factors BRIGITTE GICQUEL

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Mycobacterium tuberculosis developed various procedures to survive inside professional phagocytes and avoid the different defence mechanisms of the host. After interacting with receptors at the macrophage surface, *M. tuberculosis* is phagocytosed and remains in a phagosome that does not fuse with lysosomes and does not acidify. The exclusion of the proton ATPase and the retention of the TACO protein on the phagosomal membrane might explain this phenomenon. Also the route of entry of the bacterium into the cell might be important. Infection of macrophages by *M. tuberculosis* leads to down regulation of the antigen presenting molecules from the cell surface of antigen presenting cells. An inhibition of the synthesis of IL12, which is required for the induction of Th1 response, is also observed, as well as an inhibition of the activation of bactericidal responses by gamma interferon.

The mycobacterial factors that are responsible for above phenomena are unknown. A search for these factors was undertaken through the isolation of mutants impaired in *in vivo* growth. Genetic tools were developed enabling the transfer of DNA into mycobacteria and the inactivation of genes, either by allelic replacement or transposon mutagenesis. The construction of mutant libraries after Signature Transposon Mutagenesis (STM) allowed the screening of avirulent mutants in a mouse model. Using this technique thirteen different virulence loci were identified. Three of them are located in a cluster of genes that are responsible for the synthesis of phtiocerols and mycocerosic acid or their transport to the bacterial cell surface. In addition, putative genes coding for a transcription regulator, a lipase and transporters were found to play also a role in virulence. In

another set of experiments, the inactivation (by allelic replacement) of gene *erp*, which encodes a surface antigen, was found to lead also to an avirulent phenotype. The roles of these different gene products in virulence mechanisms still remain to be understood. However, several applications can already be considered from the identification of these virulence loci. Several of the mutants are also avirulent in guinea pigs and provide a level of protection similar to that conferred by BCG against *M. tuberculosis* infection.

0940 *M. tuberculosis* in vivo gene expression: proteomics and microarrays

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It is becoming clear that bacteria *in-vivo* express different sets of genes and flux through different metabolic pathways compared to growth *in-vitro*. Virulence, therefore, may be regarded as dependent on the correct spatial and temporal expression of specific and overlapping sets of genes in response to environmental cues encountered during the process of infection. We have developed both proteomic and transcriptomic approaches to study *in vivo* gene expression during acute and latent murine infection, as well as macrophage infection models. These include: 2D gel electrophoresis and MALDI mass spectrometry, differential subtraction hybridisation followed by library screening, RT-PCR and whole genome DNA microarrays. We have defined a limited number of genes with increased expression in acutely infected murine lungs and THP-1 macrophage cell lines using proteomics. Using the mouse Cornell model of drug induced dormancy we have detected by RT-PCR the presence of *M.tuberculosis* mRNA transcripts in culture-negative lungs, which later reactivate after steroid treatment indicating the presence of latent bacteria. This suggests that drug-induced persistent bacilli are transcriptionally active and thereby present viable targets for chemotherapy directed against the pathways used to maintain persistence. We have constructed a gene-specific, whole genome microarray for *M.tuberculosis* that has the potential to define the mRNA transcript profile of the entire genome (transcriptome). Thus microarrays, together with methods to enrich for mycobacterial mRNA from infected host tissues linked with cDNA amplification strategies, will allow *in vivo* gene expression profiles to be defined both in animal models and in human tuberculosis, even from tissues where the bacterial load is a limiting factor. The prerequisite methodology to define gene expression in *M.tuberculosis*, at the level of protein and mRNA, is now available and will enable the host-pathogen interactions to be investigated. Furthermore, the definition of *M.tuberculosis in-vivo* gene expression is likely to reveal novel targets for chemotherapeutic intervention and improved vaccines for tuberculosis.

1100 Interactions between *Mycobacterium tuberculosis* and cells of the immune system

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Mycobacterium tuberculosis is an intracellular pathogen which infects, survives and multiplies within macrophages. Since macrophages are the major effector cells for elimination of invading pathogens, *M.tuberculosis* has evolved a number of strategies for surviving in this hostile environment. Infection of macrophages by *M.tuberculosis* results in the upregulation of a

wide-range of host cell genes, particularly those involved in cell migration and the induction of pro-inflammatory immune responses. Similarly, exposure to the hostile macrophage environment results in changes in gene expression of the pathogen, enabling it to survive and multiply. This cross-talk between *M. tuberculosis* and the infected macrophage is crucial in determining the outcome of infection.

Two aspects of this cross-talk will be discussed. Firstly, the changes in macrophage gene expression will be described. Secondly, the role of a number of mycobacterial regulons in determining the outcome of infection will be described. Of particular interest is the ability of *Mycobacterium tuberculosis* to survive the low pH found within the phagosomes of activated macrophages. This appears to have been an important factor in influencing the evolution of *M. tuberculosis* as an intracellular pathogen. The ability of *M. tuberculosis* to arrest maturation of the phagosomes-lysosome pathway has been extensively studied. One effect of this maturational arrest is to reduce the acidification of the mycobacterial phagosome. Here we show that the ability to survive acid stress is one of a number of factors which influence the intracellular survival of *M. tuberculosis*.

1140 Pathogenesis and immunopathogenesis of *M. paratuberculosis*

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Paratuberculosis (Johne's disease) is a chronic granulomatous enteritis of ruminants that is caused by a member of the *Mycobacterium avium* complex, *M. avium* subspecies paratuberculosis. Many of the pathological and immunological features of paratuberculosis mirror those described in leprosy and tuberculosis. It has emerged recently that *M.a. paratuberculosis* has an extensive host range, including primates, which raises far-reaching issues about the epidemiological importance of reservoirs, potential for transmission of infection between species and the implications for control of paratuberculosis and human health. Current knowledge of the pathogenesis of paratuberculosis in ruminants and molecular biology of the causal agent will be reviewed.

1400 Early events in the lung and consequences for vaccine strategies

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The formation of the granuloma in the lungs of mice exposed to aerosol infection with tuberculosis is a complex event that takes over 100 days to fully form. The bacillus in the alveolus first encounters surfactant collectins and complement components in the local tissue fluid; absence of these molecules in gene KO mice shows that they play a minor transient role in expression of resistance. Once a site of infection is established the inflammatory process results in an influx of macrophages and lymphocytes giving rise to interstitial pneumonitis. In addition local arterioles appear to swell due to deposition of collagen, possibly creating a matrix to guide incoming T cells. As acquired immunity is expressed and the infection becomes chronic, the spatial arrangement of the granuloma becomes apparent, with aggregates of CD4 cells in the centers, and CD8 cells more peripheral [in keeping with the observation that CD8-KO mice only show differences well into the chronic phase]. It may be possible to develop vaccines both prophylactically or against these various stages in a post-exposure mode. To date the NIH screening program has tested over 190 vaccine candidates, several

of which show very promising results in the mouse and guinea pig models.

1440 Lipoproteins, glycoproteins and new vaccines DOUGLAS YOUNG

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In selecting antigens for inclusion in novel vaccines from amongst the 4,000 opening reading frames encoded within the *M. tuberculosis* genome, it is of interest to consider the potential influence of post-translational modification on immune recognition.

Post-translational acylation of mycobacterial proteins has been demonstrated by metabolic labelling and by Triton X-114 phase-partitioning. Structural analyses have not been carried out, but it is likely that mycobacterial lipoproteins are analogous to their counterparts in other bacteria in having three fatty acid chains attached to an N-terminal glyceryl cysteine residue. The characteristic consensus lipoprotein motif is present in approximately one hundred *M. tuberculosis* ORFs. Acylation has two important consequences in relation to the immune response. Firstly, bacterial lipopeptides are recognised by toll-like receptors (TLR2) on the surface of antigen-presenting cells, stimulating release of key immune mediators including IL12 and TNF α , and priming the anti-mycobacterial activity of macrophages. Acylation has also been shown to affect the subcellular location of mycobacterial proteins during infection, promoting escape from immature phagosomes and facilitating access to class I antigen processing.

Several mycobacterial proteins have been shown to be modified by glycosylation. Currently available structural evidence demonstrates attachment of short O-linked oligosaccharide chains by way of threonine residues. The physiological function of glycosylation remains to be established; a role in regulation of proteolytic cleavage has been proposed. In terms of the immunological significance, glycosylation may affect antigen processing, or influence peptide binding to antigen presenting molecules or to antigen-specific receptors.

1550 New approaches to drug design

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New drugs are needed to overcome the threat posed by the emergence of multi-drug resistant strains of *M. tuberculosis*. In addition, agents that reduce the duration and complexity of the current therapy would have a major impact on compliance and overall cure rate. A number of approaches are being taken to identify targets for novel antimycobacterial agents, ranging from biochemical studies of essential pathways to the use of genome-scale tools such as transposon mutagenesis, proteomics and transcript mapping on microarrays. Several targets have been identified and a combination of high throughput screening and rational structure-based design is being used to identify lead molecules. Researchers at GlaxoSmithKline are also working in partnership with the US National Institutes of Health in a programme aimed at identifying a drug candidate based upon the natural product thiolactomycin.

1630 Anti-tuberculosis chemotherapy: past success and future challenges

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The introduction of anti-tuberculosis chemotherapy was the first example of a randomised controlled clinical trial. This and many of the clinical trials which followed it not only provided important information about the optimal management of anti-tuberculosis chemotherapy but also raised important questions about the microbiology of the organisms and pathology of the disease. In the first trial for example patients responded rapidly but many relapsed and the organisms that were isolated had become resistant to streptomycin. Laboratory investigations of these strains demonstrated that they segregated into two resistance levels, a phenomenon which was probably due to the emergence of resistance by the two different molecular mechanisms.

By a series of clinical trials multiple drug therapy has been established and the duration has reduced from twenty four months to six. During this process the problem of persistence has been identified and in laboratory studies several models have been developed to study the shift down to anaerobic or starvation metabolism and the molecular mechanisms that accompany these physiological changes.

Although modern anti-tuberculosis chemotherapy administered in the clinical trial context results in cure in more than 98% of patients, under programme conditions - the real world - cure rates are as low as 62%. A very modest achievement when untreated tuberculosis has a 5 year survival rate of 50%. Trials of shorter regimens have been associated with unacceptable relapse rates thus improvements in anti-tuberculosis chemotherapy will result from an improved understanding of the organism and developments of new means of killing persisting organisms.

CELLS & CELL SURFACES GROUP

Microbial lifestyles

THURSDAY 13 SEPTEMBER 2001

0900 *Pseudomonas syringae* – an opportunistic plant pathogen and saprophyte controlled by specific environmental factors

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The bacterium *Pseudomonas syringae* is an opportunistic plant pathogen that can infect numerous crop plants causing devastating, economically important diseases. *P. syringae* usually causes water-soaking, chlorosis, necrotic lesions, canker, or frost injury. The species can be subdivided into more than 51 pathovars reflecting the actual host plant(s) infected. Like for other gram-negative phytopathogenic bacteria, the major pathogenicity factor of *P. syringae* is the ability to secrete virulence and avirulence proteins into the plant cells via a type III protein secretion machinery termed the Hrp (hypersensitive response and pathogenicity) system. The function of this system depends on plant-borne factors, contact with plant cell walls, and numerous abiotic environmental factors, such as pH, osmolarity, and temperature. The latter environmental parameter also seems to be of special importance for the synthesis and secretion of virulence factors, such as phytotoxins, exopolysaccharides, plant

hormones, extracellular enzymes, the ice nucleation protein, and additional other factors. For phytotoxin production and exopolysaccharide synthesis, detailed mechanisms for the thermo-responsiveness have been revealed and will be discussed. Factors important for the saprophytic fitness of *P. syringae* have also been identified during the search for thermo-regulated genetic loci in *P. syringae*. Currently, we aim at the understanding of the interplay between temperature, pathogenicity, virulence, and saprophytic survival on plant surfaces. Ultimately, our studies will lead to a better knowledge of the ecological rationale and the cellular mechanisms for thermoregulation of virulence and fitness factors in plant pathogenic bacteria.

0945 Quorum sensing as a lifestyle determinant in pathogenic and non pathogenic pseudomonads

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Quorum sensing is a regulatory mechanism generally employed by bacteria to facilitate gene expression only when the population has reached a sufficient cell density. It depends on the synthesis of low molecular weight molecules that diffuse in and out of bacterial cells. As the bacterial population density increases, so

does the synthesis of quorum sensing signal molecules and as a consequence, their concentration in the external environment increases. Once a critical threshold concentration is reached, the signal molecules activate a target sensor kinase or response regulator so facilitating the expression of quorum sensing dependent genes. The genus *Pseudomonas* contains a variety of nutritionally versatile organisms capable of adapting to life in diverse environments from, soil and water to plants and animals. In different pseudomonads, quorum sensing contributes to environmental adaptation by facilitating the elaboration of virulence determinants in pathogenic species and biocontrol characteristics in beneficial species as well as directing biofilm formation and colony escape. While pseudomonads employ a range of *N*-acylhomoserine lactone (AHL) quorum sensing signal molecules to control gene expression, certain AHLs also possess potent immune modulatory, pharmacological and antibacterial activities such that they directly facilitate bacterial survival by promoting an advantageous lifestyle within a given environmental niche.

1100 The transition of *Rhizobium* from a free-living bacterium to a microsymbiont

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The rhizobia can be divided into five genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Azorhizobium*. All of them carry out nitrogen fixation under microaerobic conditions, usually in legume root nodules although occasionally in stem nodules. There is a highly complex signalling pathway leading to nodule formation. Rhizobia detect plant flavonoids and in turn synthesise lipochitooligosaccharides, which induce nodule development in legumes. The rhizobia usually attach to plant root hairs and become trapped during root hair curling. Initially the bacteria remain inside infection threads, which grow between cells of the plant cortex. Eventually the bacteria are engulfed by cortical cells and become surrounded by a plant derived peribacteroid membrane. Bacteria undergo a maturation process in the nodule, eventually becoming mature nitrogen-fixing cells called bacteroids. Bacteroids appear to be terminally differentiated and cannot revert back to free-living cells capable of replication. The steps in formation of bacteroids are very poorly understood, perhaps largely because we have been unable to induce bacteroid formation in free-living bacteria. As a result much of our detailed knowledge of the symbiotic association between rhizobia and legumes is either ultrastructural or concerns the biochemistry of the mature bacteroid.

It is not until mature bacteroids are formed that nitrogenase, which is responsible for reduction of N_2 to ammonium, is expressed. Nitrogen fixation by the *Rhizobium*-legume symbiosis is responsible for assimilating a substantial proportion of the biosphere's nitrogen. The fixation of nitrogen by the bacteroid is energetically very expensive, with at least 16 molecules of ATP required per N_2 reduced. To sustain this requires the steady provision of a carbon and hence energy source from the plant. At its most fundamental level nitrogen fixation by the *Rhizobium*-legume symbiosis can be considered a highly regulated exchange of carbon and nitrogen. The plant receives a supply of fixed nitrogen while the bacteroid receives carbon. I will consider the substantial body of evidence that C_4 -dicarboxylates are provided by the plant for bacteroid metabolism and look in detail at the regulation of their transport and catabolism. Clearly solute

movement across the peribacteroid and bacteroid membranes is crucial to the regulation of nitrogen and carbon exchange. Nitrogen and carbon metabolism have usually been considered separately but in reality they cross-regulate each other. It has recently been shown that the secretion of fixed nitrogen by the bacteroid is more complex than once thought, with secretion of both ammonium and alanine. Synthesis of alanine may be a key regulatory step in integrating carbon and nitrogen flux in the bacteroid. As accessory pathways the synthesis of storage polymers such as polyhydroxybutyrate and glycogen may also affect carbon flux and nitrogen fixation. The overall regulation of these pathways will be considered.

1145 *Listeria monocytogenes*: life inside and outside the human host

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The gram-positive rod *Listeria monocytogenes* is a food-borne pathogen of major significance. *L. monocytogenes* is widely distributed in the environment and has commonly been isolated from foods in which it can survive and multiply. This poses a serious threat to public health as consumption of contaminated foods can result in listeriosis. The ability of *Listeria* to avoid the humoral immune system and cause disease is explained by the unusual intracellular life cycle of this organism. *Listeria* is readily phagocytosed by host cells, including epithelial cells and hepatocytes, a process mediated by internalins. The other virulence factors form a cluster on the bacterial chromosome. The transcription factor PrfA is absolutely required for the transcription of the other virulence genes of the cluster, encoding phospholipases, listeriolysin, a metalloprotease and actin polymerization factor. In addition to these virulence factors, *Listeria* appears to be well-equipped to survive a variety of adverse conditions, including low pH, acids, low water activity, low temperature, and the hostile environment of the human GI tract, which contribute to its survival in foods and to its pathogenic capabilities. *L. monocytogenes* stress adaptation mechanisms and key parameters in these processes will be discussed. An understanding of bacterial environmental sensing and responses to stresses will be essential for designing safe food processing regimes.

1400 *Myxococcus* differentiation

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See page 40

1445 Coming out on the surface: alternate life-styles of *Salmonella typhimurium*

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Swarming motility plays an important role in surface colonization by several flagellated bacteria. Swarmer cells are specially adapted to rapidly translocate over agar surfaces by virtue of their increased flagella, longer cell length and encasement of polysaccharide 'slime'. The external slime provides the milieu for motility and likely harbors swarming signals. Our laboratory has

been interested in understanding signaling pathways regulating swarmer cell differentiation and swarming motility. A recent genetic analysis of swarming in *Salmonella enterica* has suggested that the O-antigen improves surface 'wettability' required for swarm colony expansion, that the LPS core could play a role in slime generation, and that multiple two-component systems cooperate to promote swarmer cell differentiation. A model in which the external slime is itself both the signal and the milieu for swarming motility will be presented.

In contrast to the expansionist behaviour of swarming colonies, there is a prevalence in nature of microbial colonies that remain attached to a surface in associations called biofilms. Biofilms contain copious amounts of exopolysaccharides or 'slime' secreted by the adherent bacteria. Genetic studies have shown that like the swarming bacteria, single-species biofilms form in multiple steps, require intercellular signaling, and show a pattern of gene expression that is distinct from that of planktonic cells. What feature of a biofilm allows adherence in one case and expansion in another? Recent findings that shed light on this issue will be presented.

1600 Architecture and antibiotic resistance in biofilms of *Pseudomonas aeruginosa*

G.A. O'TOOLE

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Biofilms are microbial communities attached to a surface. The mature biofilm is characterized by its distinctive architecture comprised of macrocolonies surrounded by fluid-filled channels. I will describe two different mechanisms required for establishment and maintenance of this architecture.

Another well-characterized property of biofilms is their resistance to antimicrobial agents. Biofilm-grown bacteria can be up to 1000-fold more resistant to antibiotics than the same strain grown in liquid culture. We propose that some bacteria enter a resistant state that requires the expression of specific genes and gene products. It should be possible, therefore, to identify strains lacking these specific functions based on the fact that they do not develop biofilm-specific antibiotic resistance. We have identified mutant strains unable to develop biofilm-specific antibiotic resistance. The genetic and molecular analyses of these mutants will be presented. Finally, we will present a model for one mechanism underlying the increased resistance of biofilm-grown *P. aeruginosa* to antibiotics.

POSTERS:

CCS 01 Surface-associated proteins expressed by biofilm cells of *Streptococcus sanguis*

C. BLACK, M. WILSON & R. McNAB

Dept of Microbiology, Eastman Dental Institute, University College London

Streptococcus sanguis is an early colonizer of the clean tooth surface and is numerically abundant in dental plaque. Biofilm grown cells of *S. sanguis* are known to have different cell surface properties and protein expression profiles compared to their planktonic counterparts. In order to investigate in more detail the surface proteins expressed by biofilm-grown *S. sanguis*, rabbit antiserum was raised to glutaraldehyde-fixed whole cells of *S. sanguis* NCTC10904 harvested from a model biofilm device. Antiserum was used to screen a λ ZAPII expression library of *S. sanguis* genomic DNA, and 20 positive clones that reacted strongly with the antiserum were selected for further study.

Clones encoding fragments of the cell-wall-anchored adhesin CshA were encountered on several occasions. All but one of the remaining clones were identified by database search. Two main categories of protein were identified; those involved in (1) carbohydrate utilization, and (2) cell wall biosynthesis. Other clones included secreted IgA protease. The majority of clones encoded surface-associated proteins as would be expected, however some encoded proteins that were predicted (by their function and/or lack of an identifiable signal sequence) to be cytoplasmic. This work has identified a number of immunogenic surface proteins of biofilm grown *S. sanguis*. In future studies, differential expression of cloned genes will be investigated, by RT-PCR, in biofilm and planktonic cells.

CCS 02 Identification and characterisation of a cell wall associated compound from *Mycobacterium tuberculosis* and *M. bovis* BCG observed only after extended microaerobic and anaerobic culture

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Mycobacterium tuberculosis (MTB) remains a leading cause of mortality around the world. One reason for its success as a pathogen is its ability to persist and survive in the host. The mechanisms that are involved in its long-term persistence are unknown but they may be, at least in part, related to the organism adapting to the oxygen-starved environment of granulomas - early studies have shown that anaerobically cultured MTB enter stationary phase in an ordered manner. Previously, we, and others, have shown that this slowdown strongly correlates with an increased expression of the 16-kDa α -crystallin-like protein and a marked thickening of the electron dense cell wall outer-layer. Our work suggests that the compound(s) responsible for the thickened cell wall are ethanol-soluble. Here, we present our biochemical studies on this ethanol-soluble cell wall fraction from anaerobic MTB and BCG cultures up to 3 years old. We have identified and characterised a novel, pigmented, electron-dense compound that is twenty-fold more prevalent in anaerobic, stationary phase cultures than in aerobic cultures. Furthermore we show the immunomodulatory effects purified, strongly pigmented, culture filtrates from these cultures have on macrophage and fibroblast cultures *in vitro*.

CCS 03 The attachment and characterisation of chicken crop isolates and their potential for use as probiotics

CHARLENE McCANN & MARTIN COLLINS

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The widespread use of antibiotics in human and animal medicine and in animal husbandry practices has raised concerns of antibiotic resistance being passed to the human food chain. In 1998 a food safety report called for a ban on the use of antibiotics in farming as growth promoters and tighter restrictions on their use for sub-therapeutic or prophylactic purposes. Since then many poultry producers have withdrawn antibiotics in poultry feeds.

Research has indicated that many health problems in poultry may be linked to the malfunctioning of the GI tract, which has

renewed interest in the potential use of probiotics. These microbial feed supplements, which are thought to benefit the host by improving the intestinal microbial balance, may prove an alternative to antibiotics. Adhesion of probiotic bacteria to intestinal epithelial cells is regarded as a prerequisite to exert beneficial health effects. This study seeks to address the need for critical validation of *in vitro* adhesion studies and further elucidation of the microbial genes involved.

Lactic acid bacteria from the crop of broiler chickens were isolated by three methods. Subsequent DNA extractions and RAPD-PCR enabled the isolates to be grouped by their RAPD profiles. Haemagglutination of chicken erythrocytes used as an initial indication of attachment was observed for approximately 17% of bacterial isolates. Further studies will investigate the attachment of isolates to an epithelial cell line; this will be used to compare the two methods as a measure of attachment and possible uses as probiotics.

CCS 04 *spnT*, a novel gene involved in the regulation of multicellular sliding behaviour in *Serratia marcescens*

CHUN-JUN WEI¹, YU-TZE HORNG¹, HSIN-CHIH LAI^{1,2}, KWEN-TAY LUH², SHEN-WU HO^{1,2}, SIMON SWIFT⁵ & PAUL WILLIAMS^{3,4}

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Serratia marcescens SS-1 is capable of surface translocation in a flagellar independent manner termed sliding. Sliding is made possible by the production of a surfactant known as serrawettin. The production of surfactant is under the control of a N-acyl homoserine lactone (AHL) based quorum sensing system relying on the SpnI synthase and SpnR regulator. The *spnI* and *spnR* genes are convergent. Directly upstream of *spnI* is a novel gene we designate as *spnT*. Over-expression of *spnT* in SS-1 results in the inhibition of sliding and also significant cell elongation. Over-expression of *spnT* in *Escherichia coli* and *S. marcescens* CH-1 (both negative for AHL signalling) also leads to obvious cell elongation. Furthermore, over-expression of *spnT* in CH-1 inhibits flagellar dependant surface translocation (swarming) but not swimming motility. The activity of SpnT does not, however, appear to be through Spn-signalling, as both AHL and surfactant production are unaffected by the over-expression of *spnT*. Additionally, *spnT* appears to have an essential role in SS-1 as attempts to inactivate the *spnT* gene has been unsuccessful despite considerable efforts using strategies that have been used to inactivate other genes in this bacterium.

CCS 05 Erp, an extracellular protein family specific to mycobacteria

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Erp (exported repeated protein) was originally characterized as a virulence factor in *Mycobacterium tuberculosis* and was thought to be present only in *M. leprae* and members of the TB complex. Here we show that Erp is a ubiquitous extracellular protein found in all of the mycobacterial species tested. Erp proteins have a modular organisation and contain three domains: a highly conserved amino-terminal domain which includes a signal sequence, a central variable region containing repeats based on the motif PGLTS, and a conserved carboxy-terminal domain rich in proline and alanine. The number and fidelity of PGLTS repeats of the central region differs considerably between mycobacterial species. This region is however identical in all of the clinical *M. tuberculosis* strains tested. In addition, we show here that a *M. smegmatis* *erp::aph* mutant displays altered colony morphology which is complemented by all the *erp* orthologs tested. The genome sequence flanking the *erp* gene includes cell wall-related ORFs and displays extensive conservation between saprophytic and pathogenic mycobacteria.

THURSDAY 13 SEPTEMBER 2001

0905 Evolution of pneumococcal resistance and virulence determinants

CHRIS DOWSON

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Streptococcus pneumoniae (the pneumococcus) is an important causative agent of pneumonia, otitis media, meningitis, bacteremia, and a major cause of morbidity and mortality among the young, elderly, and immunocompromised. However, it is frequently carried asymptotically in the oropharynx where targets under a strong selective pressure can evolve by intra and interspecific recombination and point mutation. The past two decades have witnessed the acquisition and global spread of chromosomal and transposon-encoded resistance to all but one of the major groups of effective antibiotics. There is therefore increasing pressure to develop novel therapeutic agents. However, in order to understand fully the current spread of resistance and future evolution to novel therapies we need to look jointly at the mechanisms of resistance, the evolutionary processes involved in their acquisition and dissemination, and the population structure of this naturally transformable organism.

Population genetic analysis of isolates from carriage and disease reveals the frequent presence of dominant national, or international clones, amongst strains that are less frequently encountered. Such analysis suggests that the pneumococcal population is at linkage equilibrium, that horizontal gene transfer occurs within 'house keeping' genes, and that serotype replacement occurs in 4-6% of strains. Moreover, analysis of capsule phase variation has revealed a further mechanism for DNA rearrangement in this genetically plastic organism.

0945 Immune responses to conjugate vaccines

D. GOLDBLATT

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The success of *Haemophilus influenzae* type b conjugate vaccines and the subsequent development and licensing of *Streptococcus pneumoniae* and *Neisseria meningitidis* conjugate vaccines has focussed attention on polysaccharide antibody responses in childhood. For many years only pure polysaccharide vaccines have been available against these important pathogens. Such vaccines are limited by their inefficient stimulation of antibodies in the young, and the short lived nature of the immune response they provoke at all ages. The limitation of polysaccharides as vaccine antigens is related to the T independent nature of the immune response they induce. The absence of T cell help is associated with delayed ontogeny of the response, inefficient isotype switching, failure of affinity maturation of anti-polysaccharide antibodies and the absence of an anamnestic response on rechallenge. Large doses of some polysaccharides have also been associated with subsequent hypo responsiveness. The molecular basis for the restricted response to polysaccharides remains obscure.

The experience with conjugate vaccines has demonstrated that B cells capable of reacting with polysaccharide antigens are present in young infants and can be induced when antigen is presented in the context of T cell help. Furthermore, conjugates administered in infancy are able to induce immunological memory and are thus likely to provide long term protection. Developing

surrogates for the induction of memory (and thus indirectly the potential for long term protection) have been important in providing tools for vaccine researchers to assess the performance of individual vaccine formulations.

Recent studies of Pneumococcal 7 valent conjugate and Meningococcal C conjugate vaccine has confirmed the highly efficacious nature of this class of vaccine and holds much promise for the control of these important pathogens.

1100 *Legionella pneumophila* pathogenesis: an accident of nature?

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It is not clear how *L. pneumophila*, a ubiquitous aquatic organism not possessing a mammalian reservoir, evolved the ability to cause human disease. It is proposed that its ecology and co-evolution with lower-order eucaryotic organisms may partly provide an answer. Broadly speaking, the organism causes two diseases: mild, non-pneumonic Pontiac fever and Legionnaires' disease that is pneumonic and fatal in about 12% of cases, but can approach 50% fatality. Gross phenotypic differences occur after growth in suspension, as a biofilm, intra-protozoal or intra-macrophage. *L. pneumophila* virulence factors will be described and compared between protozoal and mammalian hosts. The role of *rpoS* in bacterial virulence and resistance will be considered in general, as will the role of type II and IV secretion systems for *L. pneumophila* in particular. The role of the ecology of this organism will be discussed in terms of environmental survival and virulence for lower-order eucaryotic organisms.

1140 The pathogenesis of *Chlamydia pneumoniae* infection

LEE ANN CAMPBELL

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Chlamydia pneumoniae causes acute human respiratory disease. Approximately 10% of community acquired pneumonia and 5% of bronchitis and sinusitis cases are due to *C. pneumoniae*. In addition, this organism has been associated with various other clinical manifestations including asthma, chronic obstructive pulmonary disease, sarcoidosis, erythema nodosum, otitis media, and coronary artery disease. *C. pneumoniae* infection is ubiquitous. In all of the geographical locations that have been tested worldwide, about 50% of adults have *C. pneumoniae* IgG micro-IF antibody, indicating past or chronic infection. Population prevalence antibody data has shown that antibody is infrequent under the age of five, increases rapidly through age 15, and then more slowly throughout the rest of life, reaching 70-80% in the elderly. Studies on the age specific incidence of acute infection have demonstrated that everyone is infected between the ages of ages 5-14. Reinfection is common. Animal models have been developed to study *C. pneumoniae* infection. Both mice and rabbits develop disease following single or repeated inoculation, which is characterized by a multi-focal interstitial pneumonia and systemic infection. In both models, chlamydial inclusions have been seen in macrophages in the lung, supporting in vitro evidence that *C. pneumoniae* can infect and survive in monocytes/macrophages. Histopathology in the lung is most severe early on and is characterized by an inflammatory infiltrate

consisting of polymorphonuclear leukocytes, which changes to predominantly mononuclear leukocytes later on in the infection and persists for longer time periods. A striking pathological feature in both models is the accumulation of perivascular and peribronchial lymphoid cells. In addition to the lung, the organism can be cultured from other anatomical sites including spleen, aorta, and peripheral blood mononuclear cells (PBMC). These findings in animal models demonstrate that infection causes systemic disease. Likewise, in humans, *C. pneumoniae* has been detected, in sarcoid skin granulomas, lung, spleen, liver, lymph node, PBMCs, and atherosclerotic lesions. Considerable attention has focused on the association of *C. pneumoniae* with atherosclerosis by sero epidemiologic studies, demonstration of the organism in atheromas throughout the arterial tree, and culture of the organism from atheromatous tissues. *In vitro* and *in vivo* studies support a putative role of *C. pneumoniae* infection and atherosclerosis. *In vitro*, investigators have shown that *C. pneumoniae* infection leads to the up-regulation of adhesion molecules and release of proinflammatory cytokines. In the presence of low density lipoprotein, *C. pneumoniae* induces foam cell formation, a hallmark of the early atherosclerotic lesion. In animal models, *C. pneumoniae* infection accelerates atherosclerotic lesion development in hyperlipidemic mice and rabbits. Large anti-chlamydial intervention studies in humans with coronary artery disease are currently underway and should provide insight as to whether *C. pneumoniae* infection contributes to cardiovascular disease in humans.

1400 *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex infections in patients with Cystic Fibrosis

ESHWAR MAHENTHIRALINGAM *PhD*

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Cystic Fibrosis (CF) is the most common autosomal recessive disease among Caucasians. Abnormalities in mucus secretion within the CF lung create an environment which is highly susceptible to bacterial infection. In early childhood respiratory infections caused by *Staphylococcus aureus* and *Haemophilus influenzae* may occur and can usually be cleared by antibiotic therapy. However, approximately 70% of CF patients eventually succumb to lung infection with *Pseudomonas aeruginosa*, which is the primary cause of morbidity and mortality. It causes chronic respiratory tract infections and is rarely eradicated as a result of antibiotic therapy. In general individual CF patients are colonized with a single *P. aeruginosa* strain which may undergo a number of phenotypic alterations as it adapts to chronic infection. Strains become mucoid (secreting large quantities of exopolysaccharide) and also become serum sensitive due to alterations in the structure of their lipopolysaccharide. *P. aeruginosa* strains recovered from chronic infection frequently down-regulate expression of virulence factors associated with motility such as the flagellum and pili. Until the recent emergence of multi-drug resistance strains, patient-to-patient of *P. aeruginosa* has not been a major problem in most treatment centres. In contrast, respiratory tract infections caused by bacteria from the *Burkholderia cepacia* complex pose a significant infection control problem. In addition, the organism is highly virulent in certain CF patients causing a rapid and uncontrollable decline in clinical condition known as “*B. cepacia* syndrome.” Because *B. cepacia* is also intrinsically resistant to many antibiotics infection is rarely eradicated. Recent examination of the taxonomy of *B. cepacia* isolates has resulted in the identification of at least seven genetically distinct species (initially designated as genomovars).

Subtle phenotypic differences between genomovars has enabled the following members of the *B. cepacia* complex to be designated as new species: *Burkholderia multivorans* (formerly genomovar II); *Burkholderia vietnamiensis* (formerly genomovar V); *Burkholderia stabilis* (formerly genomovar IV) and *Burkholderia ambifaria* (formerly genomovar VII). *B. cepacia* genomovar I, III and VI await species designation if simple differential tests can be found. Genomovar III appear to be the most pathogenic species in CF patients being associated with the majority of *B. cepacia* complex infections (~70%), poor prognosis, patient-to-patient spread, and the ability to replace existing infections with other genomovars. Outbreaks of *B. multivorans* have also been documented and this species is the second most prevalent in CF (~25% of *B. cepacia* complex infections). All the remaining species/genomovars may be recovered from CF infection, but their contribution to respiratory disease remains to be fully determined. (Dr Mahenthiralingam’s research has been funded by grants from the Canadian CF Foundation and the UK CF Trust.)

1440 Mechanisms of fungal pathogenesis in the lung

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The lung provides the portal of entry for a diverse range of fungal infections, many of which may then go on to cause disseminated life threatening diseases. A variety of virulence determinants have been implicated in the pathogenesis of these infections whilst they are in the lung. Some of these appear to be species specific, as is the case of capsule production by *Cryptococcus neoformans*, whilst others appear to be common to several genera, such as pigment production (melanization) and adherence. Thus in *C. neoformans* an anti-phagocytic capsule composed of complex carbohydrates is rapidly produced by yeasts once they are in the alveoli; although this constitutes the most obvious virulence determinant in this yeast, production of melanin via a laccase enzyme also helps to protect the fungus from immune effector cells. Pigment production also plays a role in the pulmonary pathogenesis of *Aspergillus fumigatus* infections. Inhaled conidia are protected in the lung by an ‘antioxidant shield’ produced by enzymes implicated in the synthesis of dihydroxynaphthalene melanin. Recent data now suggests that melanization is much more widespread in fungal pathogens than previously thought and the conidia of two other important fungal pathogens, *Paracoccidioides brasiliensis* and *Penicillium marneffeii* have also now been discovered to contain melanin. In the former melanization persists in the yeast form into which conidia transform in the lungs. Adherence mechanisms have been extensively characterised in *A. fumigatus* conidia, which bind to the alveolar surface; and extracellular matrix proteins such as laminin and fibronectin have been implicated as target molecules. The same molecules appear to play a role in the adherence of *P. marneffeii*. In the latter a lectin like molecule on the outside of conidia appears to recognise laminin and fibronectin. However, precise definition of the role of adherence in these fungi awaits detailed molecular investigation of the genes involved.

1550 Molecular epidemiology and transmission of *P. carinii*

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Although it was first described almost 100 years ago, the mode of acquisition and route of transmission of this enigmatic organism remains unclear.

It is firmly established that *P. carinii* is a fungus and that *P. carinii* from man and other mammalian host species shows antigenic, karyotypic and genetic heterogeneity. Cross infection between host species is not possible, suggesting host species specificity and that *P. carinii* infection is not a zoonosis. Lower levels of genetic diversity are seen in human *P. carinii* than occurs between *P. carinii* from different mammalian hosts.

The majority of healthy children/adults have antibodies to *P. carinii*, suggesting that *P. carinii* pneumonia in immunosuppressed individuals arises by reactivation of childhood, asymptomatic, latent infection. The hypothesis is challenged by the failure to find *P. carinii* in BAL fluid/lung tissue from healthy persons and in only a small proportion of HIV+ persons with respiratory diagnoses other than *P. carinii*. *P. carinii* pneumonia in man is now thought to arise by de novo infection from an exogenous source, a model supported by the findings of different *P. carinii* genotypes in each episode in patients with recurrent pneumonia.

Recent molecular data suggest that transmission of *P. carinii* from infected patients to susceptible immunocompromised individuals may occur; both within hospital inpatient units and within households (mother ↔ child). In addition, low levels of colonisation of the lungs by *P. carinii* of immunocompetent and mild/moderately immunosuppressed patients has been described – suggesting that some individuals may act as an infectious reservoir of *P. carinii*. This hypothesis is further supported by the observation that immune competent healthcare workers in close contact with patients with *P. carinii* pneumonia are more likely to be colonised with *P. carinii* than healthcare workers with minimal/no contact.

This talk will review the molecular epidemiology and transmission of *P. carinii*.

for the analysis of different fimbrial types via slide agglutination tests. Different monoclonal antibodies specific for *B. pertussis* agglutinogen 1,2 and 3 have been prepared from hybridoma cells. The frequencies of serotypes are compared between DTaP, DTP study subjects and unvaccinated matched controls. The influence of vaccine type on the serotype of *B. pertussis* are discussed and the importance of different typing reagents on the test results are also addressed.

POSTER:

CM 01 Serotyping of *B. pertussis* isolates from study participants of Pertussis Vaccine Efficacy Trial in Erlangen/Germany

S. RAMAKRISHNAN¹, P. NEWLAND² & D.X.L. XING²

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Serotype-specific agglutinogens are among the surface components of *B. pertussis* that are important factors in conferring immunity. It has been known from previous data that the fimbrial type of *B. pertussis* can change depending on the vaccination status of the population. In 1990s, a clinical vaccine efficacy study was carried out in 227 private practices in various regions of Germany in which diphtheria-tetanus acellular pertussis component (DTaP) and diphtheria-tetanus whole cell pertussis component (DTP) vaccines were administered in a double-blind randomised manner. The diphtheria-tetanus (DT) group was used as the control. A total of 79 isolates of *B. pertussis* strains were obtained from study subjects who developed the disease. These strains have been transported to NIBSC from Germany of which include control and unknown serotype. The aim of the present study is to serotype these strains using monoclonal and polyclonal antibody preparations

WEDNESDAY 12 SEPTEMBER 2001

1405 Code of Practice for the Assurance of Academic Quality and Standards in Higher Education: Postgraduate Research Programmes

CHRIS HASLAM

Assistant Director, Quality Assurance Agency for Higher Education, Gloucester GL1 1UB

The *Code of practice for the assurance of academic quality and standards in higher education* comprises of a suite of inter-related documents for the guidance of higher education institutions subscribing to the Quality Assurance Agency for Higher Education (the QAA). The *Code* identifies a comprehensive series of system-wide expectations covering matters relating to the management of academic quality and standards in higher education. In so doing, it provides a reference point for institutions as they actively and systematically seek to assure the academic quality and standards of their programmes, awards and qualifications. The *Code* assumes that, taking into account nationally agreed principles and practices, each institution has its own systems for independent verification both of its quality and standards and of the effectiveness of its quality assurance systems.

The session will explore the first published section of the *Code* relating to the support and oversight of postgraduate research study. The implications of the *Code* for the conduct of research supervisors and students will be examined.

1445 Developing supervisory competence

D. HOWARD GREEN

Chair: UK Council for Graduate Education

Dean: Graduate School, Staffordshire University, Stoke-on-Trent ST4 2DE

The Harris report, amongst others, identified the role of the supervisor in raising of quality in postgraduate research. Subsequently other agencies, including the Research Councils and QAA, have identified the need to improve supervisory skills. The practice of doing research or completion of a research degree are no longer seen as acceptable training grounds for research degree supervisors.

Training is identified as an appropriate means of developing supervisory skills. Currently training practice varies significantly across HEIs from complete absence, still all too prevalent, to accredited courses such as those of Bradford University and Leeds Metropolitan University.

Do we train all staff or just those new to supervision? Should training be compulsory? Are questions frequently asked?

Various approaches have been used to encourage staff to undertake programmes of training - from compulsion to positive encouragement. Whatever approach is adopted, to be successful, training must be part of a wider programme of support for supervisors and students and fully integrated into individual professional development.

1525 Supervisory practice at a university in the North of England: results of a questionnaire

ADRIAN R. ELEY

Division of Genomic Medicine, University of Sheffield Medical School, Sheffield S10 2RX

A questionnaire on supervisory practice was sent to 180 supervisors of 3rd year full-time UK/EU PhD students; 88 (48.8%) completed questionnaires were returned. The majority of departments had a Graduate Research Tutor and Graduate Affairs Committee or their equivalents. Unfortunately, many supervisors had not agreed with their students what their supervisory responsibilities were. Again, almost one third of supervisors had not jointly agreed with their students what was expected of the latter. The vast majority of supervisors considered that research supervision was both a teaching and a research role. Almost 90% of supervisors had not received any formal training for this role, although a significant percentage thought formal training to be a good idea. The most important area for supervisor training, perhaps not surprisingly, turned out to be the supervisor-student relationship. Almost all supervisors thought that research students should take more responsibility for their own learning, which led to my proposed concept of student self-directed learning. Other findings of the questionnaire will be discussed together with possible implications for improving supervisory practice in the future.

1625 The relationship between student and supervisor - an unequal partnership?

JAMES S. GROVES

National Postgraduate Committee of the UK, University of Lancaster, LA2 0PF

The majority of codes of practice for research study view the relationship between student and supervisor as an essentially equal partnership, founded on contract. Each has rights and responsibilities, to each other and to their institution, and each is provided with a clear method for raising grievances, usually within their department in the first instance. In many cases, however, the relationship is far from equal and can lead to exploitation of the student by the supervisor and/or the institution. The means by which supervisors are trained and, later, appraised are often ineffective and inconsistent; this can lead to the student feeling he/she is unable to raise his/her criticisms. This talk will examine some case studies in which the student-supervisor relationship has broken down, and will consider how institutions can modify their codes of practice, training programmes and appraisal systems to minimise the occurrence of such problems.

1705 Experiences of running a course for research supervisors in Sheffield

ROY JENNINGS

Division of Genomic Medicine, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2RX

In line with other universities in the UK, the University of Sheffield has given considerable thought over the past 5 or 6 years, to providing some form of rationalised and formalised guidance to those of its staff members, often but not always those staff newly-appointed to lecturer posts undertaking the supervision of research Master's and PhD students. The Graduate Office at the University of Sheffield issues a comprehensive booklet to all new graduate students, and also to all supervisors, the 'Guidebook for Research Students and Supervisors'; on a yearly basis and this is updated regularly. In addition however, via the Staff Development Unit of the

University of Sheffield, the Graduate Research Committee has initiated a 'Research Supervisor's Development Course', and this facility is available to all Departments, Division or other groupings where research students are present and working.

The course first ran in 1997, and is judged by the feedback evaluation forms, has proved both useful and interesting for staff attending the course, and particularly so for newly-appointed lecturing staff, who have been able to take advantage of several aspects of research supervision developed during the sessions, in addition to the expertise available during the course.

The aims of the programme, which is delivered either as four 2-hour lunchtime sessions, or as 2 half-day sessions, are to enable participants to develop and/or enhance effective and high quality research supervisory practices through the exploration of issues in research supervision; to set, maintain and monitor standards or provision and achievement for effective and successful research supervision, and to increase the knowledge and understanding of the supervisory and examining processes.

The contents of the programme include graduate student induction processes; the management, monitoring and assessment of graduate student progress; through years one, two and three; upgrading (M Phil to PhDs); supervisor/student interactions (with illustrative cases); conflicts, disputes and contentious issues arising during the supervisory process; the structure and process of the *viva voce* examination.

This talk describes the Sheffield Research Supervisor Development Programme in detail, and addresses some of the experiences and problems the organisers have encountered during the running and delivery of the course, and will touch upon the feedback and comments received, that have enabled the course to be improved over its 3-4 years existence.

TUESDAY 11 SEPTEMBER 2001

1410 Marine viruses and bacterial diversity - are they connected?

J. FUHRMAN

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It has become clear that marine bacteria and viruses play significant roles in global biogeochemical cycling of important elements like C, N, P, Fe, and S. Most older studies have focused on the bacteria or viruses as "black boxes" with unknown diversity, but molecular approaches have been applied recently to begin to break down these into their component parts. Bacteria and archaea are studied with cloning and sequencing of 16S rRNA genes, and rapid 'fingerprinting' techniques allow us to take a snapshot of community diversity directly from natural samples. Mixed natural virus communities can be analyzed by pulsed field gel electrophoresis to follow the diversity in genome lengths as an indicator of viral diversity. We have applied these tools to examine the relationships between bacterial and viral community diversity in Southern California ocean waters, primarily at an offshore site that is revisited regularly. We have also studied mesocosms (typically 20 to 80 liters) filled with seawater that has been manipulated by nutrient additions or size fractionation. Theory tells us that viruses can have a strong impact on bacterial diversity, because they have the potential to "kill the winner" of competition. On the other hand, viruses might simply be following changes in bacterial diversity driven by other factors. We suspect that both mechanisms operate. The data show clearly that both bacterial and viral communities change on short time scales. However, we cannot yet resolve cause and effect well enough to say when viruses drive bacterial changes or vice versa.

1455 Inorganic nitrogen utilisation - planktonic bacteria and algae slug it out

PETER J. LE B. WILLIAMS & RUBINA M.N.V. RODRIGUES
School of Ocean Sciences, University of Wales, Bangor, Menai Bridge, Anglesey

The classic paradigm of the planktonic food chain was the so-called NPZ (nutrient-phytoplankton-zooplankton) model. It has been a great favourite of the modellers despite having fundamental flaws. There have been three major shifts in understanding that have led to a move away from this simplistic model.

The paper will review these conceptual advances and to look at studies designed to resolve the dynamics based on a modern understanding of the structure of food webs.

The first shift in understanding came as far back as the 1960s, when Dugdale showed that simple arithmetic showed that zooplankton and the NPZ model in its original form considered only the larger zooplankton - the meso-zooplankton. By default the remainder of the mineralization would need to fall to the micro-organisms, but the case for this second major change to our understanding could not come until the early 1980s, when the microbiologists could make an argument for the scale of activities associated with the microbial loop. Finally, in the 1990s, the case was put together for microbial utilisation of significant amounts of inorganic nitrogen - thus bacteria were seen as both users and producers of nitrogen - particularly ammonia. The problem is

how to study this more complex foodweb. I will separate the problem into two parts - i) whether bacteria are mineralisers or assimilators of inorganic nitrogen, and where the latter how can we monitor the competition for inorganic nitrogen.

i) *To produce or to consume nitrogen*: - the classic model used to describe this is the so-called stoichiometric model, which is based on the principal that if there is more than sufficient nitrogen in the substrate for growth then the surplus will be excreted as ammonia, if in sufficient then ammonia will be assimilated. The watershed can be calculated from three properties: the C/N ratio of the growth substrate, the C/N quota for the cell, and the carbon growth yield. We have some understanding of the second two properties, but a poor understanding of the first. I will discuss an experimental approach using combined measurements of oxygen and ammonia flux which can shed light of the nature of the nitrogenous substrate.

ii) *Monitoring the scrap for nitrogen*: - conceptually this is an easier problem. It is possible to make a physical separation of bacterial from algae based on size and the tussle between two groups for the nitrogen can be teased out of measurements made on the two size fractions. I will describe a set of measurements that bear light on the competition between algae and bacteria for inorganic nitrogen.

The results show that there is a shift in the nitrogenous content of the substrate being used by the bacteria, in the mid-summer period it is nitrogen poor - giving rise to a demand for inorganic nitrogen in the post bloom period. It can be shown that at this period of the year the bacteria put up strong competition for ammonia and may accelerate the demise of the phytoplankton bloom.

1600* Interactions between marine biofilms and the zoospores of the green macrofouling alga

Enteromorpha

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Bacterial biofilms are present on most surfaces in an aquatic environment. Settlement of motile zoospores of the green alga *Enteromorpha* is influenced by these microbial biofilms. The number of zoospores settling is positively correlated with the number of bacteria present. Stimulation of zoospore settlement by biofilms was analysed for bacterial species specificity. Bacterial strains were isolated from the biofilm occurring on rocks and *Enteromorpha* thallus surfaces and identified by 16S rDNA sequencing. Single species biofilms were developed on glass slides and tested for their effect on zoospore settlement. Most of the bacterial strains enhanced zoospore settlement. Quantitative relationships between zoospores and bacterial numbers were determined. Biofilms, which stimulated settlement, were further analysed for their spatial relationship between zoospores and bacterial cells. Preferential settlement of zoospores was shown on the bacterial cells using image analysis. Stimulation of zoospore settlement by certain bacterial strains is hypothesised to be due to chemical signals produced by bacteria.

1620* Virus dynamics in a coccolithophore-dominated bloom in the North Sea

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The marine coccolithophorid *Emiliania huxleyi* has a world-wide distribution, and is well known for vast coastal and mid-oceanic blooms. It has been acknowledged that viruses are linked to the decline of *E. huxleyi* blooms. Virus dynamics were determined as part of a multi-disciplinary cruise in the Northern North Sea, to investigate the dimethyl sulphide (DMS) biogeochemistry of a coccolithophore bloom (DISCO). We used analytical flow cytometry (AFC) to determine virus concentrations through vertical profiles in the bloom. We present the first high intensity sampling data of viruses from a Langrangian survey to gain a unique insight to the temporal and spatial dynamics of viruses in an open water sight. AFC analysis of large-viruses, assumed to infect DMS-producing algae, did not appear to influence DMS/DMSP production. It is likely that microzooplankton out-competed viruses for coccolithophore prey/hosts. However, virus abundances were within the range expected for open water environments. During the langrangian experiment there was a net decrease in virus numbers suggesting that they were actively infecting hosts. An inverse relationship was observed between virus to bacteria ratio (VBR) and bacteria production. Lower VBRs were observed in a subsurface layer where concentration of bacterioplankton was higher. It was previously shown that a species of α -proteobacteria, related to the genus *Roseobacter*, dominated a bacterioplankton community that was efficiently consuming dissolved DMSP consumer in the surface and subsurface layers. The low VBRs suggested that viruses were active against this *Roseobacter* bloom however, the role of bacterial viruses in DMS/DMSP cycling was not determined and remains an area worthy of further investigation.

1640 Relations between bacterial ectoenzyme activities and polymeric algal exudates in sediments

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Benthic diatoms secrete copious amounts of extracellular polymeric substances (EPS), which predominantly comprise polymeric carbohydrates. Polymers are possibly produced as an overflow system of primary production and for motility and migration of epipelagic benthic diatoms in the sediment surface layer in response to light. Another function of EPS coatings may be to conserve water during periods of tidal exposure. Part of the EPS in sediments is readily solubilized in overlying water and leaves the sediment after tidal exposure. About the fate of EPS in sediments little is known. It is assumed that benthic heterotrophic bacteria are capable of hydrolysing the cohesive mucus-matrix and take up the hydrolysed products. Relations between benthic bacterial activities and EPS concentrations in different operational solubility and molecular weight fractions in

diatom mats suggest coupling. Increases in bacterial production coincided with increases in EPS during tidal exposure. Increases in EPS did not result in increases in bacterial ectoenzyme activities. Bacterial ectoenzyme activity (β -glucosidase activity) was inhibited in diatom mats compared to enzyme activities in sediments without mats. Inhibition of glucosidase activities may result in accumulation of polysaccharides. Depending on the cohesive properties of the EPS the sediment stability may thus increase. If true, variations in glucosidase activities by benthic bacteria may play a key role in the build-up and decay of diatom biofilms. Results and ideas are discussed in the light of recent literature.

WEDNESDAY 12 SEPTEMBER 2001

0905 Genetic diversity in the marine picophytoplankton and its relationship to bacteriophage infection

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Cyanobacteria of the genera *Synechococcus* and *Prochlorococcus* are the dominant prokaryotic components of the marine picophytoplankton and make a substantial contribution to marine primary production. We have explored the use of several different genes and intergenic regions to analyse the genetic diversity of marine *Synechococcus* strains. This has shown that there is very considerable genetic diversity within natural populations and that the extent of diversity varied markedly on a seasonal basis and also changed down the water column. This multilocus approach has also revealed that genetic exchange occurs between *Synechococcus* strains. Parallel studies on phages which infect these *Synechococcus* strains also reveal considerable genetic diversity which co-varies with that of the host. Sequence analysis of phage genomes shows that the marine phage have access to an "universal gene pool".

0950 Dissolved organic matter: the microbial nectar of the sea

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Dissolved organic matter (DOM) is the largest reservoir of organic matter and the primary source of carbon and energy for heterotrophic bacteria in the ocean. It is estimated that about 50% of the photosynthetically-fixed carbon in the ocean passes through heterotrophic bacteria in a food web referred to as the microbial loop. Given the central role of bacteria and DOM in marine carbon, nitrogen and phosphorus fluxes, there is considerable interest in understanding the cycling of this material in the microbial loop. This presentation will utilize both experimental and geochemical evidence to explore the biological origins of DOM and the mechanisms of its production and consumption. Relationships among the chemical composition and molecular size of DOM and its bacterial utilization indicate that polymeric substrates fuel much of the bacterial growth and respiration in the productive surface waters of the sea. Combined carbohydrates, such as polysaccharides, are the most abundant and reactive substrates identified in the upper ocean. Low-molecular-weight compounds of largely unknown chemical

composition are the predominant, less reactive components of DOM. There is growing evidence indicating that bacteria are a source of some of the refractory components of DOM and that the enzymatic activities of bacteria contribute to the formation of chemically-uncharacterized, low-molecular-weight DOM.

1105 C. Manna from heaven: algal-bacterial coupling in the deep-sea

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The supply of particulate organic matter, fixed by phytoplankton in surface waters, to the seabed is the major determinant of abundance and activity of deep-sea benthic bacteria.

Macroaggregates ("marine snow") and faecal pellets are the major components of the flux to the deep-sea bed, and are comprised of living and dead phytoplankton and zooplankton held together by a sticky matrix of mucopolysaccharides. These particles also contain an enriched and active population of bacteria which play an important role in the remineralization and solubilization of the particulate organic carbon, such that many aggregates will be both formed in the warmer euphotic zone and be recycled there.

However, many do escape to midwaters where decomposition rates may be reduced by the cooler temperatures and higher pressures experienced there. The reduced microbial activity on such particles may contribute to the delivery of relatively undegraded aggregates to the deep-sea bed, although there is evidence that particle solubilization by attached bacteria also helps sustain free-living bacteria in the midwaters. As many as 1×10^{12} bacterial cells/m²/year, which is equivalent to around 3×10^{13} plasmid encoded phenotypic genes/m²/year, can be transferred from the surface of the ocean to the deep-sea bed through pelagic-benthic coupling. Therefore the formation and sinking of phytodetrital aggregates may also act as a method of genetic exchange between populations previously assumed to be genetically isolated in addition to acting as an important method of delivery of essential food resources to the enormous population of bacteria found in deep sea sediments.

1150* The isolation of an antibacterial protein from the diatom *Phaeodactylum tricorutum*

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The marine diatom *Phaeodactylum tricorutum* has been shown to contain at least one antimicrobial peptide (AMP) that has been purified to homogeneity by a combination of solvent extraction and RP-HPLC. The AMP is active against a gram-positive marine bacterium. Its molecular mass, as determined by SDS-PAGE, is < 14 kDa and is currently being subjected to amino acid analysis and partial N terminus amino acid sequencing. This will be the first AMP to be fully characterised in a eukaryotic microalga and therefore provides a unique experimental system to study its expression and functional ecology at both biochemical and gene levels.

1210 Nitrogen fluxes from land to sea: the key role of algal-bacterial interactions in estuarine biofilms

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Nitrogen is the major limiting nutrient in coastal waters and enrichment of nitrogen is of concern due to increased eutrophication. Estuaries are major sites for the transformation of

nutrients flowing from the land into coastal seas. Extensive areas of sediments, rich in organic matter, provide sites for microbial transformations of nutrient loads. Key transformations with regard to nitrogen cycling are denitrification and coupled nitrification-denitrification (which both result in a transfer of nitrogen from the water to the atmosphere) and assimilation of nitrogen by benthic algal biofilms (microphytobenthos). Oxygen production by benthic algal photosynthesis (which can stimulate nitrification) and competition between bacteria and algae for pore water nitrate and ammonium results in a range of inhibitory and stimulatory effects on the sediment nitrogen cycle. This paper draws together the results from a number of research programmes in various European sites investigating the relationships between estuarine nutrient loading, rates of denitrification, sediment-water nutrient fluxes, and microphytobenthic activity. Estimates of nitrogen attenuation by denitrification in estuaries ranged between 4 – 22 % of total inorganic nitrogen load, with microphytobenthic assimilation of nitrogen increasing in significance with decreasing nitrogen loads. Nutrient budgets showed that substantial amounts of ammonium and organic nitrogen (within microalgae) are generated within estuaries. Estuarine sediments therefore both denitrify nitrate and also exchange nitrate for ammonium, a preferential nitrogen source for both planktonic and benthic algae.

1400* Algal-bacterial relationships in streams with varying DOC concentration and source

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Dissolved organic carbon (DOC) can be divided into two main components, autochthonous DOC mainly in the form of algal exudates and allochthonous DOC, namely humic substances (HS). Depending on the situation, hydrology, geology and topography of a stream or lake the relative proportions of these two components vary and previous work has shown that this can alter the nature of the relationship between bacteria and algae. This study examined the effect of HS on planktonic and biofilm communities within two streams in the Duddon catchment, UK. One stream showed a summer peak in humic-derived DOC concentration while the other stream did not. Results showed that algae and bacteria became uncoupled in the humic-rich stream, and that the HS provided bacteria with the alternative carbon source to that of algal exudates. Although this uncoupling has been widely documented in planktonic, lacustrine studies this is one of the first reports to indicate that a similar situation may occur in humic-rich streams and in the biofilms within those streams.

1420* Microbial growth and activity in the NE Atlantic deep-sea bed

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In the NE Atlantic bacteria dominate deep-sea benthic boundary layer (BBL) respiration, and are a major pathway in the decomposition of material on the deep-sea bed. The objective of this investigation was therefore to determine the rates of bacterial decomposition of natural phytodetritus and other compounds. Bacterial abundance was determined using epifluorescent microscopy and growth by the addition of ³H-thymidine. Decomposition experiments of surficial sediment, overlying phytodetritus and a 50:50 mixture of the two over a 30 day period showed significant changes in bacterial abundance and

growth. Initially the bacterial growth rate in the phytodetritus ($396 \pm 60 \text{ pmol l}^{-1} \text{ hr}^{-1}$) was significantly higher than in the sediment ($135 \pm 3 \text{ pmol l}^{-1} \text{ hr}^{-1}$) or mixture ($235 \pm 8 \text{ pmol l}^{-1} \text{ hr}^{-1}$). However bacteria in the 50:50 mixture responded rapidly, and after 4 days the bacterial growth rate was the same as that in the phytodetritus. Bacterial growth rates in the surficial sediment also increased but maximum rates were approximately a third lower than the phytodetritus or mixture. Bacteria respond to the arrival of food by producing enzymes that break it down into smaller fractions, which they use to fuel their metabolism. Prior to maximum bacterial growth rates, the activity of esterase, which is responsible for the initial breakdown of organic matter, increased rapidly and peaked at 2 days for sediment, phytodetritus and the mixture. Noticeably the esterase activity of the phytodetritus was approximately 1.5 times higher than the sediment and mixture. In addition the activity of alpha-glucosidase, which hydrolyses easily degradable storage compounds e.g. starch, also increased in the phytodetritus and mixture during the first 2 days. This suggests that the rate of bacterial decomposition of material on the sea bed is dependent on the production of exoenzymes, to initially breakdown the organic matter for incorporation, and the quantity and quality of food substrates.

1440 Marine microbial interactions and DMS production

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In 1972 James Lovelock proposed that marine emissions of dimethyl sulphide (DMS, $(\text{CH}_3)_2\text{S}$) balanced the global sulphur cycle by delivering sulphur from the sea to terrestrial environments via the air. This compound is thought to account for about 40% of the total sulphur burden of the atmosphere, and oxidation of DMS in the air produces aerosols which can act as cloud condensation nuclei and thereby influence the radiative balance of the earth. Many publications have focussed on the production and fate of DMS, from a wide range of perspectives including cell biochemistry and physiology, marine microbial ecology, aquatic and atmospheric chemistry and climate science. DMS is produced via enzymatic cleavage of dimethylsulphoniopropionate (DMSP), a compatible solute found in the cells of some marine phytoplankton. However, it remains very difficult to predict DMS fluxes to the atmosphere because DMS production involves a network of microbial interactions within the marine food web. Processes that influence DMS concentration include phytoplankton exudation, grazing, viral lysis and bacterial activity. Furthermore, recently it has been proposed that DMSP and its cleavage products DMS and acrylic acid, possibly play further roles as metabolic overflow compounds, chemical cues for attraction and deterrence, and cellular antioxidants.

1555* Community structure and diversity of Picocyanobacteria in a eutrophic lake

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Picocyanobacteria are a poorly studied group due to their small size ($< 3 \mu\text{m}$) and morphological homogeneity which makes identification and classification very difficult. They are known to

be limited by nutrients in oligotrophic waters but the controlling factors in eutrophic waters are far less clear (Voros *et al.*, 1998). This study examined the diversity within the picocyanobacterial fraction of Esthwaite Water, a eutrophic lake in the Lake District, between June and October 2000. Picocyanobacteria were found to be particularly abundant in the deep chlorophyll maxima in the upper hypolimnion, reaching densities of $8.8 \times 10^4 \text{ cells ml}^{-1}$. Picocyanobacterial abundance varied temporally as expected, but there was also significant within-lake variation. Discrimination between picocyanobacterial isolates, which appeared morphologically similar, was possible using the Hip 1 PCR typing technique (Smith *et al.*, 1998). Results showed that isolates could be clustered into distinct groups and that these groups existed in the water column at different times of the year. This suggests diversity amongst the picocyanobacteria beyond the usually recognised *Synechococcus* and *Synechocystis* and poses the question of whether the 'groups' have different ecological roles.

1615 Microbial ubiquity and ecosystem function in the aquatic environment

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Free-living microbes have unique characteristics (small size and extraordinary abundance) which appear to simplify the task of understanding the dimensions and dynamics of biodiversity at the microbial level. It is argued that:

1. All microbial species have astronomical abundance on a global scale.
2. Most, if not all microbial species ($< 1\text{mm}$) are globally ubiquitous, but detected only where they find conditions suitable for population growth, so in any natural aquatic habitat, most microbial species are typically rare or cryptic.
3. Because of ubiquitous dispersal, the global number of microbial species is relatively low, and local microbial species diversity is a large proportion of global microbial species diversity.
4. As everything (microbial) is everywhere, ecosystem functions are never limited by lack of microbial diversity.

POSTERS:

EM 01 An investigation into the occurrence of F+ specific bacteriophage in Scottish shellfish

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In the UK shellfish harvesting sites are classified sites according to their levels of *Escherichia coli* (an indicator of the level of faecal pollution) in accordance with EU directive 91/492/EEC. However, it is now well documented that shellfish considered suitable for human consumption using the *E. coli* standard might contain harmful human viruses. Incorporation of a viral indicator in the form of F+ specific bacteriophage has been suggested but is not yet established. In this study, shellfish samples from 116 Scottish sites were simultaneously analysed for *E. coli* and F+ specific bacteriophage. *E. coli* levels were enumerated using a five

tube, three dilution MPN method with confirmation using chromogenic agar. F+ bacteriophage levels were evaluated using the host bacterium *Salmonella typhimurium* WG49 in a standard double agar overlay method with the origin (human or animal) of the viruses determined using oligoprobes. The majority of samples had undetectable levels of F+ specific bacteriophage and there was little correlation between *E. coli* and F+ specific bacteriophage levels in positive samples. This may be indicative of the remote and hence sparsely populated locations of the majority of shellfish production areas in Scotland. However, F+ specific bacteriophage were found in 9 % of shellfish samples from Class A sites indicating the presence of human viruses at these sites where shellfish can be sold directly for human consumption.

EM 02 Influence of nutrient limitation on abundance and composition of extracellular carbohydrates produced by the marine diatom *Cylindrotheca fusiformis*

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Although the presence of marine snow is common in marine ecosystems, the massive production and accumulation of large pelagic mucous aggregates such as those observed in the Northern Adriatic Sea is unprecedented. The mucilage phenomenon has recently occurred with increasing frequency, exerting detrimental effects on tourism, local fisheries and benthic ecosystems. There is still no general agreement on the causes of formation of the aggregates and the main organisms involved in the process, but increasing evidence from field and laboratory studies indicates that diatoms, especially *Cylindrotheca sp.*, might play a significant role in shallow coastal waters. We performed a laboratory investigation using 'batch' cultures of *Cylindrotheca fusiformis*, aimed at evaluating the role of P- and N-starvation, and different N:P ratios, on extracellular carbohydrate production and composition. The cultures were grown for 21 days in 5 different nutrient conditions: nutrient replete (f/2) and nutrient deplete media (f/2 with 1/3 P, 1/6 P, 1/3 N and 1/6 N), with a N:P of 24.3, 73.0, 145.9, 8.1 and 4.0, respectively. Sampling was performed weekly for nutrient analysis and chemical and physico-chemical analysis of extracellular carbohydrates, while cell growth was followed daily by using a hemocytometer counting chamber. Total and dissolved carbohydrates were analysed by colorimetric method. The macromolecular features of exopolysaccharides produced in the cultures were investigated by isolation and fractionation using gel permeation chromatography (GPC). Monosaccharide composition of fractions and of original samples were analyzed by gas chromatography techniques.

The concentration of total carbohydrates (TCHO-C) in the medium reached at day 21 was similar in the different treatments, ranging from 1.02 mmol C L⁻¹ in 1/3 P to 0.87 mmol C L⁻¹ in 1/6 N. Conversely, per cell production of extracellular carbohydrates varies significantly, with maximum TCHO-C in the 1/6 P cultures (1.71 μmol C · 10⁶ cells) and minimum TCHO-C in the control (0.68 μmol C · 10⁶ cells). A similar trend is observed for both particulate and dissolved carbohydrates, with P-limitation determining a higher release of organic C per cell compared to the control and the N-limited cultures. Analysis of the monosaccharide composition of the exudates shows galactose to be the major component of the saccharidic exopolymers (37-42%). In P-limited conditions we found an increase of galactose

content and a simultaneous decrease of glucose, while the remaining saccharide composition was almost unaffected. Finally, the overall sugar composition of these exopolymers, especially that in the severe P-limiting condition, is remarkably similar to the one obtained on natural mucilage samples collected in the Northern Adriatic Sea during the 2000 Summer event of massive aggregation.

EM 03 Effects of the extracellular products released by *Mycobacterium tuberculosis* on the development of *Euglena gracilis*

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It is well known that Vitamin B complex favours genre *Euglena* development and organisms belonging to *Mycobacterium* genre synthesised these kind of substances. Due to these facts and taking into account they can share the habitat in nature, we decided to make different assays to prove interactions between these groups. For these purposes we use *Euglena gracilis* MAT isolated from a polluted river and *Mycobacterium tuberculosis*, cultured together in our laboratory. We selected this taxa of *Mycobacterium* as it has shown the largest production of vitamins. We assayed two different conditions of *Euglena gracilis* cultures, one liquid medium and the other solid medium. Different concentrations of the extra cellular products (EP) of *Mycobacterium tuberculosis* were added to *Euglena gracilis* cultures. The growth of the algae was estimated by cellular number per millilitres of culture and by chlorophyll production. Both parameters showed a positive relation when *Euglena gracilis* was cultured in liquid medium. In solid medium, we tested two different assays: a) *Euglena gracilis* was spread out over solid medium in the Petri dishes and it was added a disc containing a determined concentration of EP; b) it was added a disc over the solid-medium containing a determined concentration of EP and an estimated number of *Euglena gracilis* cell. It was observed positive and negative –an inhibition ring was formed– interactions in these latest studies. Chemical studies of the EP are being carried out to find those substances involved in these interactions.

EM 04 Comparison of microbiology, filter media and plant communities within constructed wetlands treating wastewater containing heavy metals

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The aim was to investigate the treatment efficiency of passive vertical-flow wetland filters containing different plant communities (predominantly *Phragmites Australis* and/or *Typha Latifolia*) and granular media with different adsorption capacities in order to save capital costs, to produce high quality effluent, and to investigate the microbial interactions in the filters. Gravel, sand, granular activated carbon, charcoal and Filtralite (light expanded clay) were applied. Lead and copper sulphate were added to polluted urban beck inflow water in order to simulate pre-treated mine wastewater, landfill leachate or highway runoff and to test the toxicity of lead and copper to wetland biota. The interactions between growth media, microbial and plant

compositions and the reduction of predominantly lead, copper, biochemical oxygen demand and potentially pathogenic bacteria were investigated. The mean lead concentrations were 1.281 mg/l and 0.028 mg/l for inflow and outflow water, respectively. The mean inflow and outflow concentrations for copper were 1.114 and 0.199 mg/l, respectively. The average BOD₅ (5-day biochemical oxygen demand) was 3.2 mg/l for inflow and 0.8 mg/l for outflow water. However, after maturation of the biofilm, which dominated the top sediment, all wetlands reduced the heavy metals sufficiently and performed statistically similar (71~98% for Log₁₀ data sets). It follows that there is no additional benefit in using adsorption media to enhance filtration performance.

EM 05 Planktonic microbial activity in the vicinity of salmonid mariculture cages

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Salmonid mariculture is now a major industry in coastal environments and results in enhanced inputs of organic nutrients to local waters from fish feed and excreta. Such inputs may have a detrimental effect on the local environment and have been implicated in the occurrence of harmful phytoplankton blooms; however, the impact of these nutrient inputs on the structure and function of the pelagic ecosystem is still poorly understood. The aim of this study was to investigate the effect of mariculture activity on the bacterioplankton of a Scottish sea loch, and assess the degree to which bacterioplankton production may be controlled by protozooplankton grazing pressure. Experiments were undertaken during summer months at two stations at differing proximity to mariculture cages in Loch Fyne. Dissolved and particulate organic carbon and nitrogen concentrations were broadly similar at both stations, as was the biomass of bacterioplankton and protozooplankton. By contrast bacterial production tended to be greater at the station closest to the cages. Protozooplankton grazing removed >100% of bacterioplankton production at the station further away from the cages but only removed 24 – 77% of production at the station closest to the cages. The implications of these findings are discussed.

EM 06 Searching for a rapid sensitive indicator of contaminant stress on a marine bacterium

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Marine bacteria drive major biogeochemical cycles, have rapid life cycles and respond quickly to environmental changes. We investigated the use of these organisms as bio-indicators of contamination in seawater. Representatives of some contaminants typical from coastal waters were tested: a toxic hydrocarbon (Phenanthrene), a pyrogenic hydrocarbon (Pyrene), a pesticide (Lindane), a detergent (Linear Alkylbenzenesulphonate), a flame retardant (TBBA) and a metal (copper). The stress of these contaminants on *Vibrio natriegens* NCIMB 587 was studied during short time series experiments (usually up to 12-72 hours), looking at selected indicators: changes in cell concentration (optical density of the cultures), growth rate, respiration (CTC fluorescence), protein and DNA synthesis ([³H]leucine and [³H]

thymidine uptake), and cell wall composition (lectin binding). Bacterial indicators revealed clear symptoms of stress when *V. natriegens* was exposed to copper: all cellular activity ceased immediately after inoculation. Complex changes occurred during the exposure to the detergent: after an initial instant reduction in bacterial numbers, from 10⁹ to 10⁸, and respiration, both indicators increased, though bacterial numbers never achieved those of control; in contrast, protein synthesis was inhibited throughout the experiment whereas DNA synthesis was stimulated in the first 24 hours. Pyrene caused a 46% average increase in respiration of *V. natriegens*, but otherwise growth and production remained unchanged. Lindane caused a transient stress on bacterial growth and respiration that, however, normalised after approximately 10 hours. No effect was observed in any of the bacterial indicators when exposed to Phenanthrene. The metal and the detergent provoked a toxic stress on the bacteria, which can be evaluated studying the effect on the cellular indicators. The effect of other contaminants was less clear.

EM 07 Regulation of microbial nutrient turnover by the green alga *Caulerpa*

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Interstitial water concentrations of dissolved nitrogen and phosphorus are several times higher in the rhizosphere of the green alga *Caulerpa* than in the surrounding sediment. The alga releases photosynthetic products into the substratum stimulating the sulfate-reducing bacterial community. In addition to remineralizing organic N, many sulfate-reducing bacteria are capable of dinitrogen fixation. Both processes make available ammonium for plant production. Under the strong reducing conditions that prevail in the rhizosphere of *Caulerpa*, precipitated phosphate is dissolved and carbon dioxide is released. Thus, *Caulerpa*-bacteria associations couple all of the major nutrient cycles, making possible prolific nutrient turnover and growth on eutrophicated substrata. Bacterial populations associated with *Caulerpa*'s rhizosphere were identified and localized by fluorescence in situ hybridization. These populations and their activities appear dependent upon nutrient concentration. Under high sediment nutrient loads, the activities of certain bacteria located in the rhizosphere of *Caulerpa* are reduced. As nutrient loads fall, sulfate reducing and ammonium oxidizing bacteria within the rhizosphere become more active. Experiments are in progress to determine if the bacterial floras associated with the rhizoids of *Caulerpa* are environmentally dependent. Activation of bacterial communities by growth of *Caulerpa* may enhance remediation of eutrophicated or otherwise polluted sediments.

EM 08 Protozoan grazing rates on surface-associated prey

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Protozoa are considered major grazers of bacteria and algae in the plankton and one would assume that the situation is equivalent, if not greater, in biofilms due to the high abundance of prey species within them. However, the availability of prey to protozoa in biofilms is complicated as the prey may take on many forms i.e.

suspended cells, loosely attached cells and those which are embedded in the gelatinous matrix. Information on which prey-forms are susceptible to protozoan grazing and which are potentially afforded refuge is therefore required.

This poster describes a method for determining protozoan grazing rates on a surface-associated bacterium i.e. a GFP-expressing *Escherichia coli*. Upon ingestion by a protozoan, a combination of acidification and digestion within the food vacuole leads to an irrecoverable loss of GFP fluorescence in the prey. As protozoan grazing proceeds, a decrease in fluorescence over time is recorded which equates to a protozoan grazing rate. Grazing rates obtained for different protozoan species show that a significant fraction of loosely attached and embedded bacteria can be removed from surfaces by protozoa over short periods of time.

EM 09 Bacterial isolation and characterization from flora associated with the toxic dinoflagellate

Alexandrium catenella

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A clone of the toxic dinoflagellate *Alexandrium catenella* (ACC01) present in the South of Chile, synthesises several analogues of saxitoxin and has been associated with paralytic shellfish poisoning. We isolated and identified three accompanying bacterial strains. These were isolated by inoculation of dinoflagellate cultures in Difco Bacto Marine Agar. Three phenotypically different bacterial colonies were isolated and purified. The identification of each strain was done by PCR amplification of their 16S rRNA genetic loci of approximately 1500 base pairs. The method involves the use of universal priming sequences such as 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3'. The amplified product was sequenced and compared with the nucleotide sequence data of GENBANK using BLAST search. The sequences analysis suggested the most probable affiliation of two strains to a subclass of ?-Proteobacteria and one strain to a subclass of ?-Proteobacteria. The ?-Proteobacterias were phylogenetically closer to (AF022407.1) *Pseudoalteromonas* sp (94%) and (AF124521.1) *Ruegeria atlantica* (98%) both not previously associated to *A. catenella*. The ?-Proteobacteria was related to (AF235114.1) *Cytophaga* sp (92%), previously associated to *A. catenella*. The role of these strains on the growth and/or toxin production of *A. catenella* ACC01 is currently under investigation.

EM 10 The isolation of *Mycobacterium spp* from groundwater, chlorinated distribution systems and biofilms

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Mycobacterium sp are well established in all types of aquatic systems ranging from groundwater to potable water and raw water. As with a lot of outbreaks of waterborne illnesses they are usually prevalent as a result of treatment deficiencies. However, findings within potable water have revealed that these organisms have been resistant to the normal chlorination processes. Experiments have shown that chlorine levels of less than 1.0 mg/l may not inactivate a number of these opportunistic pathogens. These include *M. fortitum*, *M. gordonae*, *M. avium*, *M. chelonae*, *M. kansasii* and *M. intracellulare*. Research has also found that *Mycobacterium* sp are more resistant to ozone and chloramines than *E. coli*. Evidence of the growth of mycobacterium in biofilms, due to the persistence of certain strains of Mycobacteria in potable water (studies have found this to be as long as 41 months) is evident, particularly during summer months. Growth in biofilms has also been reported in old portions of piping and sections of piping that experience corrosion problems and elevated pH. Some regrowth has also been reported in dead-ends, evident in water distribution systems, where low chlorine levels are evident and total organic carbon and turbidity are high. *Mycobacterium* are also very prominent in buildings plumbing systems where water flow is very slow.

We are convinced of an association of *Mycobacterium paratuberculosis* (MAP) and Crohn's Disease, which is a chronic inflammatory disease of the gastrointestinal tract of humans. MAP is present in faeces of infected cattle and may be present in water supplies in areas where infected faeces wash into the water supply. We are presently investigating the survival of MAP in domestic hot water supplies to explore whether Crohn's Disease may be acquired from water and inevitably biofilms containing MAP organisms.

We also review in this paper the methods employed in the detection of *Mycobacterium* spp in biofilms and drinking water which have, and still are taking place, in the UK and USA.

EM 11 *Mycobacterium palustre* sp. nov., a new slow-growing mycobacterium isolated from clinical, veterinary and natural water specimens

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Taxonomic studies were performed on a phenotypically homogenous group of 13 mycobacteria isolated from clinical, veterinary and stream water samples. The methods applied included chromatographic analyses of bacterial lipids, biochemical tests, and sequencing of 16S rDNA and ITS1 region. Positive results in urease, Tween hydrolysis, and pyrazinamidase tests, and a negative result in semiquantitative catalase test, combined with an ability to grow at 42 °C distinguished this group from other yellow pigmented slow-growing mycobacteria. Unique fatty acid and mycolic acid profiles in chromatographic analyses, and the results of gene sequencing indicated an earlier undescribed

mycobacterial species for which the name *Mycobacterium palustre* sp. nov. is proposed. According to 16S rDNA sequencing, *M. palustre* is phylogenetically closest to *Mycobacterium kubicae*, a recently described species. One of the strains was isolated from a lymph node biopsy of a child with cervical lymphadenitis. Thus *M. palustre* should be listed among potential inducers of pediatric cervical lymphadenitis. The veterinary isolates originated from lymph nodes of slaughter pigs. The majority of the strains were recovered from natural waters, which highlights the role of the environment as a source of potentially pathogenic mycobacteria. The type strain E846^T was isolated from a stream water in Finland.

EM 12 Molecular detection of *Mycobacterium bovis* and related species in soil

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A study was done to determine the survival and distribution of *M. bovis* in the environment.

To determine if *M. bovis* survives and remains viable in soil, samples from a farm site in Ireland, which had undergone a bovine tuberculosis outbreak one month prior, were studied. Molecular techniques were used to detect *M. bovis*, PCR, using oligonucleotide primers directed to three *M. bovis* group specific "antigen" genes, MPB64, MPB70 and Esat6 was carried out. A system to quantify the number of viable *M. bovis* organisms in the samples was established. RNA was extracted from the soils, then RT-PCR carried out and subsequent short round PCR, followed by Southern hybridisation and computer-aided image analysis of the blots. This gave a method to quantify the number of cells present.

To determine the diversity of the total mycobacterial population in these soils, genus specific 16S rRNA gene primers were developed and used to provide products which were then cloned and sequenced. This revealed the presence of other important pathogenic *Mycobacterium* species including *M. avium*, and *M. paratuberculosis*.

EM 13 A conserved genetic module that encodes the major virion components in both the coliphage T4 and the marine cyanophage S-PM2

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Cyanophages (viruses that infect cyanobacteria) are abundant in the marine environment and are thought to be a significant factor in determining the population dynamics of the unicellular phycoerythrin-containing cyanobacteria of the genus *Synechococcus*. Sequence analyses of a 10 kb region of the genome of the marine cyanophage S-PM2 have revealed a homology to coliphage T4 that extends as a contiguous block from gene 18 to gene 23. The order of the S-PM2 genes in this region is similar to that of T4, but there are insertions and deletions of small ORFs of unknown function. In T4, g18 codes for the tail sheath, g19 the tail tube, g20 the head portal protein,

g21 the prohead core protein, g22 a scaffolding protein and g23 the major capsid protein. Thus, the entire module that determines the structural components of the phage head and contractile tail structures are conserved between T4 and this cyanophage. The significant differences in the morphology of these phages must reflect the considerable divergence of the amino acid sequence of their homologous virion proteins, which uniformly exceeds 50%. We suggest that the enormous diversity of phages in the sea could be a result of genetic shuffling of such commonly shared modules. These conserved sequences could facilitate genetic exchange by providing partially homologous substrates for recombination between otherwise divergent phage genomes. Such a mechanism would thus expand the pool of phage genes accessible by recombination to all those phages that share common modules.

EM 14 Development of virus-specific probes to determine the influence of *Emiliania huxleyi* viruses on biogeochemical cycling

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Emiliania huxleyi is a marine coccolithophorid, which has a world-wide distribution. It is well known for vast coastal and mid-oceanic blooms. Its production of calcium carbonate coccoliths and its role in CO₂ cycling and dimethyl sulphide (DMS) production makes *E. huxleyi* an important species with respect to past and present marine primary productivity, sediment formation and climate, and a key species for current studies on global biochemistry cycles. DMS accounts for most of the sulphur entering the atmosphere over the open oceans and its atmospheric oxidation products are the major source of sub-micron aerosol particles over remote marine areas. Viral induced lysis of phytoplankton is a likely alternative route for DMS/DMSP release and an area of research not well understood. To investigate the role viruses have on biogeochemical cycling, a number of *E. huxleyi* viruses (*EhV*) were isolated during the later stages of the much-publicised *E. huxleyi* bloom off the coast of Plymouth during July 1999. The viruses were shown to have large double stranded DNA genomes of about 400 kbp in size. Phylogenetic analysis of the DNA polymerase genes of these viruses suggests that *EhV* belongs to a new genus within the family of algal viruses, *Phycodnaviridae*. In addition, *EhV*-specific DNA polymerase primers were designed and used to study the algal virus diversity during an *E. huxleyi* bloom. Future work will involve combining DMS production data with changes in the composition of viruses throughout the progression of an *E. huxleyi* bloom to gain invaluable insights on the role of viruses in sulphur cycling.

EM 15 Temperature induction of viruses in symbiotic dinoflagellates

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Bleaching manifests itself as a loss of symbiotic dinoflagellates (zooxanthellae) and/or chlorophyll from a variety of symbiotic hosts, including corals and sea anemones. Bleaching is known to

result from a range of environmental stresses, the most significant of which is elevated temperature, and how these stresses elicit a bleaching response is currently the focus of intense research. One consequence of environmental stress that has yet to be considered is viral attack. Here we have isolated a transferable infectious agent believed to be a virus, from zooxanthellae of the temperate sea anemone *Anemonia viridis*. The infectious agent is induced by elevated temperature. Once induced, the filterable agent can be further propagated without heat induction, thus fulfilling Koch's postulates. We propose that zooxanthellae harbor a latent viral infection that is induced by exposure to elevated temperatures. If such a mechanism also operates in the zooxanthellae harbored by reef corals, and these viruses kill the symbionts, then this could contribute to temperature-induced bleaching.

EM 16 Adaption of the dilution experiment to quantify viral induced phytoplankton mortality

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There is an increasing body of evidence that viruses are as significant as microzooplankton in the control of phytoplankton and bacterial production in the marine environment. Previously, no single technique has ever been used to quantify both grazing and viral induced mortality of aquatic microbial populations. The objective of this investigation was to adapt the Landry and Hassett dilution technique so that it yielded a coefficient of viral induced mortality in addition to a coefficient of grazing. This was accomplished by setting up parallel dilution experiments, one according to the original protocol that creates a gradient of grazing pressure and a second adapted to create both a gradient of viral concentration and grazing pressure. Water for the experiments was taken from 11 m³ mesocosms filled with adjacent fjord water during a mesocosm experiment in Raunefjorden, 20 km south of Bergen, Norway. A bloom of autotrophic picoeukaryotes developed in the mesocosms and as the bloom began to crash the coefficients of grazing ranged between 0.27 and 0.6 d⁻¹ and coefficients of viral induced mortality between 0.11 to 0.3 d⁻¹. The results demonstrate that the dilution technique can be successfully adapted to measure viral induced mortality. Furthermore, the data reveals that viruses can be as important as microzooplankton in the control of picoeukaryote blooms.

EM 17 Detection of marine myanophages: A SMART approach to discriminate between different strains

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Ecological studies of algal viruses require reliable, specific techniques for detection, quantification and analysis of diversity. These would allow some understanding of the effect of viruses on bloom dynamics. However, many of these techniques lack the specificity which ecological studies of algal viruses require.

Molecular techniques have been instrumental in changing our understanding of the composition of virus communities. A novel isothermal amplification assay has been developed to detect and discriminate specific DNA or RNA targets. The amplification process, which does not require thermal cycling or involve copying the target sequence, uses two target-specific probes to generate large amounts of an RNA signal. A colorimetric system then detects and quantifies the signal. The assay; SMART (Signal Mediated Amplification of RNA Technology), is simple to perform and when fully developed will be suitable for high throughput screening. SMART probes were designed for two strains of marine cyanophage and tested on synthetic targets, purified cyanophage genomic DNA and crude cyanophage lysates. The assay was shown to be highly strain specific in these systems. We are currently optimising the assay for use on natural samples and to detect cyanophage RNA in infected host cells. By developing a bank of SMART probes it will be possible to detect a range of viruses in seawater samples, so gaining an insight into marine virus community structure.

EM 18 Growth of mixed cultures of *Paracoccus denitrificans* and *Desulfovibrio desulfuricans* in gel-stabilized gradient systems

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Sulfate reducing bacteria are a ubiquitous group found in numerous anaerobic habitats ranging from the human guts to ocean sediments, though, occasionally, as opportunistic pathogens, they may cause serious illnesses. The degree of anaerobicity of these organisms has been the subject of various investigations, and in this study, a simple gel-stabilized gradient system was employed for such a purpose.

Desulfovibrio desulfuricans (anaerobe) and *Paracoccus denitrificans* (aerobe) were grown separately and together in 250 ml beakers containing gel-stabilized lactate gradient systems. These and the control sets were incubated under the atmosphere of air or oxygen-free-nitrogen for seven days at 30C. Oxygen, pH and Eh were measured at various depths of the gels by needle electrodes. Viable counts, sulfide, lactate and acetate were determined in slices from cores removed from each gel.

The sulfate reducer (*D.desulfuricans*) could only survive long exposures to oxygen in the presence of the aerobe (*P.denitrificans*). Physico-chemical measurements correlated with bacterial population activities. Overall indirect evidences suggest that *D. desulfuricans* could occupy anoxic micro-niches within the oxic zone of aerobically grown mixed cultures.

MONDAY 10 SEPTEMBER 2001

0900 M³C = Measurement, Monitoring, Modeling and Control of biotechnological processes

BERNHARD SONNLEITNER

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The demands in modern biochemical engineering sciences with respect to measurement and control do change substantially. As the title of this presentation - which is also the title of a new Working Group following the previous Working Party of the EFB - says, essentially both on-line monitoring and modeling need to be pushed besides classical measurement techniques and control approaches and respective activities need to be forced.

After a short review of what has been achieved in this area during the past decade, major issues for the future will be raised and should pave the way for a lively discussion. The major R&D challenges brought forward - what is state-of-routine and what state-of-the-art, what needs to be tackled, where are priorities, and why - are the following:

- Bioprocess performance monitoring and fault detection
- Efficient experimental design issues in view of increasing importance of continuous (and probably integrated) bioprocesses
- Modeling of bioreactors and entire bioprocesses in a reliable and robust way, probably including molecular dynamics
- Fight "data drowning" by highly automated data selection and processing
- Monitoring and detection of impurities and contaminants, including early identification and back-tracing of process failures
- Faster product/process innovation and development to increase economic competitiveness
- Reconfiguration control and integrated design to increase efficiency and reliability [quality management]
- Biological waste (water) treatment [environmental remediation through bioprocess engineering] and ways to reduce wastes in bioprocessing.

0935 Measurement and control of nutrients and by products using a biosensor based analyser

N. RIMMER

YSI, Hampshire

Researchers studying biotechnology processes have found that the concentrations of key nutrients and by-products can affect the overall health and productivity of their processes. By regulating these nutrients and by-products to low, but constant levels, they have been able to improve cell lifetime and productivity.

A biosensor-based analyser can be used to measure, quickly and accurately, up to two such variables simultaneously. Measurement options include glucose, lactate, ethanol, methanol, choline, lactose, galactose, glutamine, glutamate and sucrose. The analyser operates by using an immobilised enzyme to convert the analyte of interest to hydrogen peroxide, which is then measured electrochemically by a platinum electrode.

The analyser pulls samples directly from a bioreactor through an externally mounted solenoid valve and pump. The solenoid valve switches between the sample stream and antiseptic, assuring sterility for up to six months. The analyser then compares the measured value with the target value previously

entered by the user. Any difference is used in a PID algorithm to control the pump dispensing the relevant nutrient or diluent. The system has been shown to control nutrient or by product levels to within 10% of the user's set point.

1010 Complex cell culture

D. MAINWARING

Lonza, Slough

Abstract not received

1115 NIR spectroscopy applied to fermentation monitoring and control

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Near infra red spectroscopy is a technology which has only recently been applied to the bioprocess industry with a view to monitoring and control of bioreactions. The technology allows the rapid measurement of analytes (substrates, biomass and products) in complex, multi-component fermentation broths. In addition NIRS is non-invasive, requiring no sample preparation and can be used in a number of different modes (off-line, at-line and on-line) depending on the process requirements.

This paper will look at models developed for several microbial bioprocesses (*Pichia pastoris*, *Streptomyces fradiae*, *Escherichia coli*) and animal cell cultures (CHO-K1) and indicate how the models can be used to improve control of the processes.

1150 Automatic generation of bioprocess models for monitoring and control

CHRIS TAYLOR

Adaptive Biosystems Ltd, Luton

Mathematical models have been shown to be useful in monitoring and control of bioreactors. Recent work has favoured data-driven empirical techniques, such as Neural Networks, over more traditional mechanistic models derived from first principles. Inferential sensors based on Neural Networks have been used in academia and industry to give online readings for variables which can normally only be measured offline, and to improve control of key parameters; empirical techniques have also been used to aid experimental planning and speed up process development.

Typically, developing useful models requires a large investment of time and brainpower from a skilled modeller to come up with something that will work. Data-driven techniques have gone some way towards automating this process, but it still requires time and energy to make something useful and robust. This is a problem if, for example, you want to be able to readily generate working models for a variety of processes in as short a time as possible, without having to employ armies of mathematicians. Most of the powerful software tools available for this type of modelling are toolkits, so that it still requires lots of skill and effort to build a working system.

This talk will outline some recent work aimed at going further down the route of automated model generation. The eventual aim is models that effectively 'build themselves' given good quality data and some information on the organism and process. While this Utopian situation is still some way off, newer techniques

based on evolutionary computation promise more sophisticated ways of searching parameter space. Combined with hybrid modelling techniques, which can incorporate existing scientific knowledge, they have the enormous potential as practical tools for bioprocess enhancement.

1400 combination of electroanalysis and chemometrics for bioprocess monitoring

CONRAD BESSANT

Centre for Analytical Science, Cranfield University at Silsoe, Bedfordshire MK45 4DT

A recently developed methodology that combines the speed and simplicity of electrochemical detection with the power of chemometrics will be described for on-line monitoring of organic compounds. The new method uses non-specific voltammetric detection at a single unmodified metal electrode to elicit information-rich responses from a sample without prior separation. Multivariate calibration is then employed to extract individual analyte concentrations from this response. A case study involving the simultaneous determination of glucose, fructose and ethanol will be used to demonstrate that the new technique enables the rapid determination of multiple analytes (including aliphatics, which are traditionally difficult to detect) in complex mixtures to an accuracy comparable to that of other methods.

1435 Optimisation of fed-batch fermentations using hybrid models

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Significant improvement in fermentation performance can be achieved by implementing advanced monitoring and control procedures. Provided a representative process model is available it can be used in fault diagnosis, performance estimation and prediction and scheduling. An important area where models can be used to great benefit is in the optimisation of performance. In batch and fed-batch bioprocesses, the maximum performance is achieved by optimising the initial conditions and subsequent profiles of manipulated variables such as feed rates, temperature and pH during the process operation.

Although there are many subtle variations in model form, in general models belong to one of two categories: mechanistic (Paul et al, 1998) or empirical (Warnes et al, 1996). Empirical models are generally structured and do not take account of the physiological behaviour. Mechanistic models, on the other hand, attempt to approximate these characteristics in mathematical form. Since both of these model types have their limitations, a combination of both - the so-called hybrid models (Schubert et al, 1994, Fu and Barford, 1996) - have been argued to offer most benefit by combining the strengths of both modelling approaches.

The main objective of this contribution is to determine the strategy for optimising the feed profile of a penicillin fed-batch fermentation process. In order to achieve this, firstly a model of the process is required. Here, a serial hybrid modelling approach has been adopted. Subsequently, the Chemotaxis random search

optimisation procedure (Montague and Ward, 1994) was used to optimise the feed profiles. This procedure has been applied to two case studies, industrial and lab-scale fed-batch penicillin fermentations. A serial hybrid modelling structure, consisting of an ANN followed by a mechanistic model, was adopted in both case studies. In the industrial case study, the outputs of the ANN are specific rates for biomass and product formation that are fed to a simple unstructured mechanistic model. In the second case study additionally the DO₂ prediction was included in the hybrid model structure. The incorporation of the DO₂ into the model acts as a constraint on total productivity in the optimisation procedure. The developed hybrid models were then used for off- and on-line optimisation of process performance by feed manipulation. In both optimisation procedures, the Chemotaxis algorithm was applied to determine the parameters of the polynomial(s) describing the feed profile(s) that achieved this optimum. The experimental verification of the proposed on-line optimisation procedure was then tested on the lab-scale fermentation.

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1540 Chemical sensors applied to fermentation processes

WILLI RAKOW

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It is widely acknowledged in the fermentation industry that control of microbial cultures in bioreactors is often sub-optimal, and, that one major contributory factor to this sub-optimal control is a lack of real time information about what is occurring within the bioreactor.

However, recent developments potentially allow better insights into bioprocesses. Promising in this context is this new approach of a non-invasive on-line bioprocess monitoring technology - the electronic nose technology. Chemical sensor systems (i.e. electronic noses) are used to extract information from the composition of volatiles emitted from the cell culture.

This presentation gives an overview about the latest developments in the area of solid-state chemical sensors and their application to bioprocess monitoring. The presentation is focused on the process how to derive information from the sensor signal that finally leads to a potential increase of yield and reproducibility of the bioprocesses.

1615 Fermentation monitoring for efficient downstream processing

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The effect of upstream operations upon the efficiency of downstream processing and the final product yield is widely acknowledged. However, no articles in the scientific literature to date attempted to link explicitly the performance of fermenters with the efficiency of downstream processing. Most of the research effort in bioprocess optimisation, modelling and supervision concentrated on individual unit operations, mainly fermenter monitoring (Glassey et al, 1997, Gregersen and Jorgensen, 1999). Although some authors attempted to link the performance of seed fermentations and/or raw material quality to fermentation production (Ignova et al, 1999, Cunha et al., 2001) downstream processing is usually excluded from such optimisation efforts. This may however reduce the overall process efficiency, especially in the case of recombinant protein production. In these systems it is widely recognised, that forcing the host system into excessively rapid rate of product formation or high levels of accumulation may cause inclusion body formation and thus lower the yields of active final product. Therefore, a holistic view of bioprocess optimisation is required to increase the overall process yield.

This contribution aims to highlight the methodology used to assess the impact of fermentation upon the downstream efficiency for an industrial recombinant human albumin production. Multiway Partial Least Squares Regression (MPLS) and Radial Basis Function (RBF) artificial neural networks were applied to quantitatively estimate the product yield initially at individual stages of the process. Subsequently models have been developed on data from fermentation and centrifugation estimating the purification yield from a critical chromatographic downstream processing steps. The need for careful data selection and preprocessing will be highlighted and a methodology for successful overall bioprocess optimisation suggested.

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1650 Measurement of dissolved carbon dioxide using an *in-situ* fibre optic chemical sensor

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High levels of dissolved carbon dioxide have been shown to inhibit cell growth, nutrient utilisation and product formation in fermentation and cell culture processes.

An *in-situ* fibre optic chemical sensor offers an alternative to traditional ways of monitoring this variable, such as off gas analysis and off line measurement with a blood gas analyser.

The sensor operates on the Severinghaus principle and contains a pH sensitive dye, hydroxypyrene trisulfonic acid. Dissolved carbon dioxide diffuses across a gas permeable membrane to produce a change in this dye. The change is then measured optically via a fibre optic cable.

The sensor can be steam sterilised before the start of a fermentation run and is calibrated at a single point against a reference gas before use. It has been shown to measure dissolved carbon dioxide over a 0-25% range to an accuracy of 5% of the reading or 0.2% absolute, whichever is the greater.

WEDNESDAY 12 SEPTEMBER 2001

0900 Evolution and diversity of ds DNA phages

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It is estimated that there are approximately 10^{30} dsDNA phages on earth. The completed genomes sequences of about 50 completed phage genomes are available. Comparisons have demonstrated similarities and extreme diversity between phages that infect similar host bacteria. Most surprising however are the relationships that are not predictable on the basis of the evolutionary distance of the hosts as connections can be made between phage genomes that infect extremely diverse bacteria. These connections are not between whole genomes, rather at the single gene level where individual genomes show extensive mosaicism. Taken together these observations suggest that dsDNA phages have access by horizontal DNA exchanges to a global gene pool.

0945 Functional genomic analysis of a novel antibiotic resistance gene transfer system

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SXT is an unusual mobile genetic element that was originally isolated from *Vibrio cholerae* O139, the first non-O1 serogroup of *Vibrio cholerae* to cause epidemic cholera. This 100kb conjugative genetic element encodes resistances to the antibiotics sulphamethoxazole, trimethoprim, chloramphenicol and streptomycin. Despite the fact that this element is self-transmissible, no autonomously replicating form of the element has been isolated. Instead, SXT integrates site specifically into the chromosome in the 5' end of *prfC*. SXT encodes an *int* gene with similarity to the ϕ integrase family and the integration and excision mechanisms of SXT shares features with ϕ . A circular extrachromosomal form of SXT is generated by recombination of the left and right ends of the integrated element and this circular form of SXT appears to be a requisite intermediate for its transfer. SXT can mobilize certain plasmids in trans as well as chromosomal DNA in an Hfr-like manner. Unlike its self-transfer, mobilization of plasmids and chromosomal DNA is not dependent upon excision of the element from the chromosome. SXT or closely related elements are currently present in most clinical O1 and O139 *V. cholerae* isolates derived from Asia.

We determined the DNA sequence of SXT. The 100kb SXT genome had a modular organization. A 17kb composite transposon-like element contained all of the antibiotic resistance genes of SXT. The transfer genes bore homology to F-like plasmids and were organized into several likely operons. There were several genes with sequence similarity to bacteriophage derived genes as well. Interestingly, nearly half of the putative open reading frames lacked any similarity to sequences in GenBank. We used a one-step gene replacement system (PNAS, 97:6640-6645, 2000) to analyze the functions of nearly every open reading frame present in SXT. These studies allowed the determination of genes required for SXT transfer, excision, integration and regulation as well as a cis-acting sequence that

constitutes the origin of transfer. Although the SXT transfer-related genes are similar to those of F-related plasmids, the circuitry governing regulation of transfer is similar to lysogenic bacteriophages. Both excision and transfer functions were controlled by the same activator. Interestingly, this activator represents a fusion of *flhC* and *flhD*, the two master activators of flagellar transcription. An SXT encoded repressor of the λ family regulates the activity of this activator. Akin to induction of lambdaoid prophages, agents such as mitomycin C and certain antibiotics that activate the SOS response were found to stimulate SXT transfer. Thus, our studies suggest that SXT is a chimeric element combining properties of conjugative plasmids and lysogenic bacteriophages. Finally, this work suggests that the use antibiotics may promote the dissemination of antibiotic resistance genes present on such elements.

1100 Pathogenicity islands and IS elements in the evolution of bacterial virulence

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Mobile genetic elements, such as insertion sequences, transposons, phages, and genomic islands, are common components of microbial genomes. Together with point mutations, homologous recombination, and horizontal gene transfer, mobile DNA elements play a major role in the generation of novel genetic and phenotypic variants of microorganisms. In the past few years, these processes have been intensively investigated in a variety of human pathogens and, in most of these studies, specific regions carrying virulence-associated genes have been identified. These large chromosomal elements (up to 200 kb in size) have been termed "pathogenicity islands" (PAIs). They represent a subgroup of genomic islands and preferentially encode adhesins, toxins, iron-uptake systems, capsules, secretion systems and other virulence factors. Similar to genomic islands, PAIs are instable chromosomal regions that are flanked by repetitive DNA sequences with both aberrant G+C content and codon usage. PAIs are frequently associated with tRNA loci and, in most cases, carry (cryptic) integrase loci, IS elements or other "mobility factors". PAIs have been described in more than 20 Gram-negative and Gram-positive bacteria. It has been suggested that the clustering of (mainly virulence-associated) genes on these specific genetic elements and their mobilization may strongly facilitate both insertions and deletions of huge amounts of genetic information.

Insertion sequence elements (IS) are equally important constituents in the genome of pathogenic bacteria. They represent small mobile DNA units that mediate all the functions required for their own mobilization. In recent studies, we have investigated the impact of a specific IS (i.e. IS256) in the generation of phenotypic and genetic diversity in the nosocomial pathogen *Staphylococcus epidermidis*. Epidemiological studies revealed that the element is widespread among multiresistant isolates from clinical origin. IS256 reversibly inactivates the *icaADBC* operon which encodes enzymes responsible for biofilm synthesis and participates, therefore, in phase variation of this essential virulence factor. However, as a result of transposition, IS256 can also enhance the expression of adjacent genes by forming strong hybrid promoters. Furthermore, the element gives rise to complex

DNA rearrangements in *Staphylococcus epidermidis* which can also be detected *in vivo* during an infection. Experiments on the transposition modus of the element gave evidence that IS256 transposes by an alternative transposition pathway which is characterized by the formation of transposon circles. Our data suggest that IS256 plays a crucial role in genome flexibility and thus contributes considerably to the rapid phenotypic and genetic adaptation of *Staphylococcus epidermidis* to changing environmental conditions.

1145 The Rag Locus of *Porphyromonas gingivalis*
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Porphyromonas gingivalis, an anaerobic Gram-negative bacterium, is one of the leading aetiological agents of periodontal disease, a chronic inflammatory condition of the soft and hard tissues of the tooth attachment apparatus. The Rag locus in the chromosome of this organism appears to have a significant influence on pathogenicity. Insertional inactivation of individual *rag* genes has a major effect on virulence in a murine lesion model and detection of this locus by PCR of clinical samples is correlated with the extent of periodontal destruction at the sample site. Initial genetic characterisation of this locus revealed the presence of two co-transcribed genes, *ragA* and *ragB* (for receptor antigen A and B) which appear to have arisen via horizontal gene transfer on the basis of a lower G+C ratio to the remainder of the *P. gingivalis* chromosome, evidence of potentially mobile insertion sequences flanking this operon and a restricted distribution in laboratory and clinical isolates of this species. RagB is a major immunodominant outer membrane antigen of unknown function whilst RagA has sequence similarity to TonB-linked receptors in other Gram-negative bacteria. On the basis of these data we proposed that the Rag locus represents a novel pathogenicity island in *P. gingivalis* which may encode an outer membrane protein complex involved in either extracellular ligand recognition and transport or signal transduction both of which would be dependent upon a TonB-related system of cytoplasmic membrane and periplasmic proteins. Interrogation of the unannotated *P. gingivalis* genome has identified an orf upstream of *ragA* with regional similarity to ExbD, one of the cytoplasmic membrane proteins that energise the TonB system, and subsequent PCR analyses have confirmed that this orf is co-distributed with the *rag* genes in clinical isolates. Hence the Rag locus may have the coding potential for a complete system of proteins required for ligand transport/signal transduction in this organism. More recent investigations have demonstrated that this locus is present in the same chromosomal location in all strains including those of diverse PFGE type suggesting a single acquisition event. However there is considerable between strain heterogeneity at this locus indicative of insertions/deletions which may have a bearing on the virulence of *P. gingivalis* in periodontal disease.

1400 The contribution of horizontal acquisition in the evolution of virulence in *Neisseria* and *Helicobacter*
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Gene transfer between species is important in the evolution of virulence. In addition to genes carried by mechanistically mobile

elements a significant proportion of this process is achieved by natural transformation in several species. Such sequences can often be detected on the basis of unusual sequence composition. We have modified 'dinucleotide signature analysis' to analyse longer component sequences and to address single open reading frames. Analysis of *H. pylori* and *Neisseria* spp. with these new tools reveal several new features that are informative about the recent evolutionary history of these pathogens and their acquisition of host-interactive, virulence associated proteins. These analyses reveal that the majority of such horizontally acquired genes are not located within 'pathogenicity islands' and frequently lack any of the traditionally associated features of these elements. These new methods have facilitated identification of many recently acquired genes that have not been recognised using previous methods and have also facilitated more detailed analysis of previously described regions, such as the 'cag' pathogenicity island of *H. pylori*. This approach when applied to the analysis of related genomes also suggests the presence of insertional 'hotspots'. The theoretical framework for the identification of these genes, the results of these gene specific studies, and the repertoires of the genes that are identified will be discussed.

1445 Mobile elements and horizontal transfer in the mycobacteria
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Analysis of mycobacterial genomes has revealed a number of surprises relating to mobile elements and horizontal transfer. The genome of *Mycobacterium tuberculosis* H37Rv contains 56 loci with similarity to insertion sequences, with IS belonging to the IS3, IS5, IS21, IS30, IS110, IS256 and ISL3 families. Strikingly, the IS1552 element showed a high level of identity with an IS from *Rhodococcus opacus*, suggesting horizontal transfer. The genome also contains two related prophage with similarity to *Streptomyces* phage. A novel repeated sequence, the REP13E12 family, is present in 7 copies on the *M. tuberculosis* chromosome. This sequence appears to contain a phage attachment site and may be responsible for the variable location of prophage in *M. tuberculosis* isolates. Comparative genomics has shown that *M. tuberculosis* contains multiple examples of interkingdom domain fusions, indicating previous horizontal transfer events. Examination of the *Mycobacterium leprae* genome has also revealed the putative transfer of an aminoacyl-tRNA synthetase from a eukaryote. Mycobacterial genomics is clearly offering new clues both to genome dynamics and to the evolution of these most successful pathogens.

1530 Plasmid evolution-lessons from *Yersinia pestis* and other genome sequences
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Yersinia pestis is a flea-transmitted systemic pathogen responsible for the pandemic zoonosis bubonic plague. Population genetics shows it to be a clone which has recently emerged from the enteric pathogen *Y. pseudotuberculosis*. Acquisition of two plasmids pPst (10 kb) and pFra (100kb) carrying essential virulence factors is likely to have been a key element in this evolutionary leap from enteropathogen to insect-

associated systemic pathogen. Unexpectedly, genome sequencing shows over 58% of the *Y. pestis* pFra plasmid is more than 97% identical to pHCM2, a cryptic plasmid from a recent clinical isolate of *Salmonella enterica* serovar Typhi, another species of Enterobacteriaceae which is an exclusively human pathogen. Various sequence features suggest pHCM2 is more likely to have been the progenitor plasmid of the sequence in common: over-representation of the canonical *E. coli* Chi sequence seen throughout pHCM2 and only in the common regions of pFra, numerous bacteriophage T4 gene homologues in pHCM2 which are only partially represented in pFra. pFra seems to be the product of cointegration of pHCM2 with an unknown replicon carrying the F1 capsular operon encoding a major virulence factor. Chi over-representation shared by pFra, and the *Salmonella* plasmids pHCM2, pHCM1 and pR27 is an unexplained feature of these large enterobacterial plasmids.

POSTERS:

MI 01 Amino alditols as inhibitors of mycobacterial cell wall biosynthesis

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Antibiotic resistance is now presenting a serious challenge in the treatment of infectious diseases. As the prevalence of multi-drug resistant strains of bacterial pathogens such as *Mycobacterium tuberculosis* increases, there is an increasing need for identifying new antimicrobials and antimicrobial targets. One such target is UDP-Galactopyranose mutase (UDP-Gal mutase) which is an enzyme that interconverts UDP-Galactopyranose (UDP-Galp) and UDP-Galactofuranose (UDP-Galf). The unusual sugar Galf is a component of the cell wall of a number of pharmacologically interesting microorganisms including *M. tuberculosis*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. By inhibiting the enzyme involved in the interconversion of UDP-galactopyranose (UDP-Galp) and UDP-galactofuranose (UDP-Galf) then, as galactofuranose is not found in man, a selective target against the bacterium will be found.

Herein we report a number of amino alditols that have been shown to inhibit both UDP-galactopyranose mutase and enzymes involved in the rhamnose biosynthetic pathway. In addition, some of these compounds have shown whole cell activity against both *M. tuberculosis* and *M. smegmatis* but have no cytotoxicity against mammalian cells. (*This work is supported by the BBSRC, EPSRC, NIH, The Wellcome Trust and GSK-Action TB Program*)

MI 02 A set of *M. tuberculosis* proteins expressed in response to isoniazid treatment

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Isoniazid is thought to exert its bactericidal action on mycobacteria by inhibiting the biosynthesis of cell wall mycolic acids, thereby rendering the cell susceptible to reactive oxygen radicals and other environmental factors. A recent DNA microarray gene expression study on *M. tuberculosis* identified a cohort of genes that are upregulated in the bacterium immediately after exposure to isoniazid. A cluster of genes thought to be involved in mycolate biosynthesis and fatty acid metabolism, and one gene known to be involved in free radical detoxification (the alkyl hydroperoxide reductase *ahpC*) were among those identified as being upregulated. A further set of genes of unknown function were also found to be upregulated. Our hypothesis is that the proteins encoded by this upregulated cohort of genes may be involved in the protective mycobacterial response to the toxic consequences of isoniazid activity. We have screened the isoniazid-induced cohort of genes using a variety of bioinformatics techniques in order to choose those proteins most suitable for structural analysis using X-ray or high-field nuclear magnetic resonance (NMR) techniques. We have selected four genes of unknown cellular function that are immediately suitable for further structural study, amplified them from *M. tuberculosis* genomic DNA using the polymerase chain reaction and expressed them in *E. coli*.

MI 03 Identification of mycobacteria-specific NK-like CD8⁺ cells in neonatal calves

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Experimental trials with BCG in cattle have shown variable protection against challenge with virulent *M. bovis*. Pre-exposure to environmental mycobacteria, and the induction of an inappropriately biased immune response, has been suggested to be a major factor affecting the efficacy of BCG vaccination. Vaccination of neonates that have not been exposed to environmental mycobacteria could circumvent this problem and may be an appropriate means of immunising against *M. bovis*. We have investigated the response of neonatal calves to mycobacteria using dendritic cells as antigen presenting cells. We report that, in calves as young as 1 day old, there is a population of CD8⁺ cells that respond to mycobacterial antigens. These CD8⁺ cells respond to BCG infected dendritic cells by proliferating and by producing IFN γ . The CD8⁺ cells were comprised of both CD3⁻ CD8⁺ (NK-like) and CD3⁺ CD8⁺ T cells and the response was shown to be non-MHC restricted. In gnotobiotic calves the presence of CD8⁺ cells that responded to mycobacteria infected dendritic cells declined with age and responses were found to be maximal between 1 and 2 weeks after birth. However, in conventionally reared calves BCG reactive CD8⁺ cells were observed only at 1 day of age and not at 1 week or 3 weeks of age. There was no evidence for CD4⁺ T cell activation at any time in any animal tested. The presence of mycobacteria reactive, IFN γ secreting, CD8⁺ cells in immunologically naïve neonatal calves may have important consequences for the induction of strong Th1 biased immunity upon antigenic challenge.

MI 04 Contribution of CD8⁺ T-cells to immunity to *Mycobacterium bovis* in cattle

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Two groups of six calves were inoculated intratracheally with 10⁴ virulent *M. bovis* AF2122/97. Ten days after inoculation calves in one group were depleted of CD8⁺ cells by injection of specific monoclonal antibodies (mab) for 7 consecutive days. The second group of calves was inoculated with isotype matched control mab. Effectiveness of depletion was confirmed by flow cytometric analysis of blood samples and lasted for up to 2.5 weeks. Post-mortem examinations performed six weeks after challenge inoculation revealed widespread tubercular lesions in both groups with no obvious differences between CD8 depleted and control calves. Animals depleted of CD8⁺ cells had higher concentrations of IFN γ in sera, detected by ELISA, compared to control animals. No differences were detected in the isotype of the specific IgG response in sera to mycobacterial antigens. Likewise, no differences between the two groups were detected in the proliferative responses *in vitro* to mycobacterial antigens or in IFN γ production from blood cultured *in vitro*. Thus, while no apparent role in gross pathology could be attributed to CD8⁺ T-cells, these cells appear to play a role in the production of IFN γ *in vivo*, which would be expected to have consequences for the bias of the immune response, following infection, and potentially the pathogenesis of disease in the longer term.

MI 05 Measurement of drug resistant tuberculosis using a quartz crystal based DNA biosensor

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Diagnosis of drug-resistant tuberculosis is time consuming, with current culturing methods taking approximately 12 weeks. A significantly faster method would be beneficial for its early diagnosis and treatment. We have used the quartz crystal microbalance (QCM) to develop a viability test for *Mycobacterium smegmatis*, with the aim of developing a rapid diagnostic test for drug-resistant strains of *M. tuberculosis*.

The QCM is able to detect mass and viscoelasticity of surface-absorbed species in the liquid phase. In this way, the resonating crystal can measure DNA hybridisation events. DNA probes are immobilised within the shear-wave-sensing region of the crystal. The subsequent highly specific binding of target DNA can be measured as a decrease in resonant frequency or a change in crystal impedance characteristics.

Messenger RNA (mRNA) has been used as a measure of viability because of its relatively short half-life in comparison to rRNA and DNA. The gene targets chosen were *SigE* (for its ability to be induced) and 23S (as it is present in multiple copies within the cell). These genes are also present in both *M. smegmatis* and *M. tuberculosis*. The DNA probes were single-stranded oligonucleotides that were homologous to either *SigE* or 23S. The sensing surface was modified with a variety of synthetic polymers. The probes were then immobilised on these layers and hybridisation events monitored.

MI 06 A continuous culture model for studying dormancy in *Mycobacterium tuberculosis*

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Tuberculosis kills three million people per year and approximately one third of the world's population is infected with *Mycobacterium tuberculosis*. A key to this bacterium's success is its ability to persist within the body for years in a dormant or latent state primed for reactivation. Understanding the physiology of dormant tuberculosis and the molecular mechanisms co-ordinating latency is an important goal for mycobacterial researchers. This information will allow the development of new anti-TB drugs targeted to latent bacteria. Experimental studies on latent tuberculosis have been hampered by a lack of appropriate experimental models. The Wayne's method for dormancy in tuberculosis has proved a very useful model for studying the dormancy response in a batch culture system. It was the aim of this project to develop a continuous culture system for mycobacterial dormancy. *M. bovis* BCG was grown in the chemostat at graduated oxygen levels and also at different growth rates to simulate the conditions of latency. Various parameters associated with latency were then examined, including stress, antibiotic resistance and anaplerotic metabolism (isocitrate lyase activity). It was found that the chemostat culture system provided a controlled and defined environment to study latency and is a useful dormancy model for *Mycobacterium tuberculosis*.

MI 07 The construction of a recombinant BCG strain for use as a vaccine against the cattle parasites

Plasmodium falciparum and *Fasciola hepatica*
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As well as being widely used as a vaccine for tuberculosis, BCG is also a promising vector with which to introduce foreign antigens to the immune system. Recombinant BCG strains expressing antigens for a range of disease have been constructed and used with varying degrees of success to elicit immune responses in animal models. Such vaccination strategies have the advantages that BCG is a powerful adjuvant whilst also being safe, easy and cheap to administer. This study describes the construction of a BCG strain carrying antigens for the cattle parasites *Plasmodium falciparum* and *Fasciola hepatica*. The vaccine strain is required to have a several gene knockouts, each generated by a series homologous recombination events either side of the gene and counter-selection with *sacB*. To permit the selection of recombinant BCG without using antibiotics, *leuD* has been deleted to generate a leucine auxotroph that will be complemented by constructs containing genes for the parasite antigens. In addition, a number of genes for antigenic proteins from BCG are being deleted to allow the development of a multi-immunodiagnostic tests that will differentiate animals vaccinated with the recombinant BCG strain from those with naturally occurring *M. bovis* infections.

MI 08 Generation and characterisation of potential virulence gene knockout mutants of *M. tuberculosis* H37Rv

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One of the most interesting group of potential virulence genes identified in *M. tuberculosis* genome are those that make up the four *mce* operons. Each *mce* operon contains eight genes that are predicted to encode a similar set of proteins with characteristic signal sequences or hydrophobic residues. Previous studies (Arruda S et al, 1993. Science 261:1454-1457) have demonstrated that *mce1* is involved in promoting entry of the pathogen into host cells. The function of the other *mce* genes is obscure, as is the reason for the four operons; although comparative genomics has demonstrated that *mce3* is absent from avirulent *M. bovis* BCG.

To examine the function of the *mce* genes, we have generated a series of knockout (KO) *mce* mutants of *M. tuberculosis* H37Rv by homologous recombination. To date we utilised allele exchange to construct unmarked mutants with a deletion of the entire *mce3* and *mce4* gene operons (10kb) as well as a double *mce3* and *mce4* mutant in *M.tuberculosis* H37Rv. The phenotype of these KO mutants is currently being investigated.

MI 09 Enhancing the protective efficacy of DNA vaccines against *Mycobacterium bovis*

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In recent years, bovine tuberculosis (TB) has re-emerged as an important disease of cattle in the UK. Despite a sustained program of testing of cattle herds for TB, initially introduced in the UK after the Second World War, the disease persisted at low levels in some parts of the country. Over the last 15 years there has been a progressive increase in incidence of the disease in the affected areas, with almost 1,000 herds affected last year. A recent scientific review concluded that further control measures are needed to tackle this problem, and that the best prospect for future control is to develop a vaccine to protect cattle against TB.

Previous studies had shown that a DNA vaccine that encodes the *M. bovis* antigen MPB83 protected mice against challenge with virulent *M. bovis*, and induced immune responses in vaccinated cattle. A variety of strategies have emerged to try to improve the immunogenicity and protective efficacy of DNA vaccines, including co-immunising with genes encoding cytokines.

In order to improve the immunogenicity of the MPB83 DNA vaccine, we have analysed the effect of co-delivering plasmids encoding bovine cytokines that promote a Th1 biased immune response, which is known to be central to immunity to TB. We show that compared to MPB83 DNA alone, co-immunisation of cattle with a plasmid encoding IL-12 or IL-18 increased specific IFN γ production by cultured lymphocytes. No further enhancement was observed following co-immunisation of both cytokine-encoding plasmids in conjunction with MPB83 DNA.

These findings suggest that co-immunisation with plasmids encoding appropriate bovine cytokines may be a means of increasing the immunogenicity of DNA vaccines against *M. bovis*.

MI 10 Effects of *Mycobacterium tuberculosis* *rpf* homologs on susceptibility of mycobacteria to antimicrobial agents

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Micrococcus luteus cells have recently been found to secrete a resuscitation promoting factor (Rpf) that stimulates the growth of culturable and resuscitation of dormant *M. luteus* cells. Rpf also stimulates the growth of several mycobacteria. Analysis of the *Mycobacterium tuberculosis* genome revealed 5 predicted ORF's with significant homology to Rpf, leading to speculation that Rpf-like proteins may play a role in controlling the growth of mycobacteria and reactivation of clinically latent tuberculosis. The ORF of each *M. tuberculosis* homolog has been cloned downstream of the *hsp60* promoter in *pmv261* and overexpressed in *M. smegmatis* and *M. bovis* BCG. The growth properties of these recombinant strains have been investigated. Overexpression of three homologs has been found to stimulate growth and one homolog appears to suppress growth; differences in colony morphologies have also been observed with prolonged incubation. The susceptibility of the recombinant strains to anti-tuberculosis drugs has been investigated and one Rpf-like gene appears to increase the susceptibility of *M. smegmatis* to rifampicin and results in a lower MIC of drug for this strain. These results indicate that expression of the *M. tuberculosis* *rpf* homologs may have a significant effects on growth and drug susceptibility in mycobacteria.

MI 11 The randomness of IS6110 insertion sites in *Mycobacterium tuberculosis*

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Introduction: The main source of genomic polymorphism in *Mycobacterium tuberculosis* is attributed to IS6110. Due to its varying copy number and assumed random distribution, IS6110 RFLP is the internationally recognised typing method for this organism. In addition, these IS6110 insertions into ORFs create natural mutants, which can be used to study the biology of *M. tuberculosis*. We are investigating the spatial distribution of the IS in the *M. tuberculosis* genome to *i.* quantify the randomness of transposition target site selection at the nucleotide, regional and genomic level, *ii.* investigate the extent of consensus in the sequences at the target sites, *iii.* assess the significance of insertions in particular regions. **Methods:** We have sequenced the IS6110 insertion sites from 160 clinical isolates obtained from geographically dispersed regions. **Results and Discussion:** In the study, 322 unique insertion sites were identified. Although insertions were distributed throughout the genome, some regions more often contained IS6110 than would be expected by chance.

MI 12 Comparative analysis of the *bfp-per* region of enteropathogenic *Escherichia coli* adherence factor plasmids

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Enteropathogenic *Escherichia coli* (EPEC) are an important cause of diarrhoea, particularly among children in developing countries.

In order to better understand differences in virulence gene expression among EPEC, we conducted hybridisation, PCR and sequence analysis studies within the bundle-forming pilus – plasmid-encoded regulator (*bfp-per*) region of the EPEC adherence-factor plasmid of representative strains. The *per* genes were found in 96.8% of strains while the cryptic *trcP* gene was present in only in O86 and O111 strains and one O142 strain. The sequence immediately upstream of the *per* promoter region in a subset of strains was replaced by an IS1294-homologous sequence and in most of these strains, the *bfp* operon was truncated. In a minority of strains -- O119:H2, canine and a subset of O128:H2 and O142:H6 strains, which are deficient in virulence gene expression, frameshift mutations in *perA* leading to premature truncation and consequent inactivation of the gene were identified. In all other strains, the C-terminal helix-turn-helix motif of PerA was 100% conserved. The gene content of EAF plasmids varies among EPEC strains and conserved deletions and frameshift mutations provide an explanation for reduced virulence gene expression levels and lower epidemiological impact of atypical EPEC strains.

MI 13 Characterization of a novel pathogenicity island encoding neuraminidase (*nanH*) among toxigenic *Vibrio cholerae* isolates

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Horizontal transfer of genes encoding virulence factors has played an important role in the evolution of *Vibrio cholerae*. The genes encoding cholera toxin (*ctxAB*), the main cause of profuse secretory diarrhoea in cholera, are encoded on a filamentous bacteriophage CTX Φ and its receptor the toxin-coregulated pilus (TCP), is also encoded on a mobile genetic element known as VPI. In this study the gene encoding neuraminidase, an enzyme that acts synergistically with cholera toxin, was mapped within a pathogenicity island (PAI), the percentage G+C content (42%) was considerably lower than that of the entire *V. cholerae* genome (47%). The island, designated the *Vibrio* pathogenicity island 2 (VPI-2), has all the characteristics of a PAI including the presence of an integrase gene and insertion at a tRNA locus. PCR and Southern hybridisation analysis were used to screen *V. cholerae* O1, O139 and non-O1/non-O139 strains for the presence of VPI-2. All toxigenic *V. cholerae* O1 serogroup isolates contained a complete VPI-2 whereas most non-toxigenic isolates lacked the island. Out of 14 toxigenic O139 isolates tested, only 1 contained the entire island. This strain, MO2, was isolated from India in 1992. The remaining 13 strains, isolated in India between 1992 and 1996, lacked *nanH* and all open reading frames upstream of the gene. Interestingly, we found that VPI-2 was also present in *V. mimicus* isolates, which suggests the possible mobilisation and horizontal transfer of this region between strains.

MI 14 Mobile genetic elements among *Vibrio cholerae* natural isolates

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In this study, we examined isolates of *Vibrio cholerae* the causative agent of the diarrhoeal disease cholera for the presence

of a number of mobile genetic elements which encoded virulence factors. Epidemics of cholera are caused by two *V. cholerae* serogroups, O1 or O139, of the more than 200 serogroups identified. *V. cholerae* isolates belonging to non-O1/non-O139 serogroups, which can be isolated in abundance from aquatic or estuarine sources, cause sporadic cases or limited outbreaks of diarrhoea in humans. The *ctxAB* gene which encoded cholera toxin (CT) resides in the genome of CTX Φ , a filamentous bacteriophage that infect *V. cholerae*. The CTX Φ receptor, the toxin-coregulated pilus (TCP), a bundle-forming pilus is encoded within the genome of another filamentous bacteriophage VPI Φ . We found by PCR and Southern blot analyses that the presence of CTX Φ corresponded with the presence of VPI Φ . Upstream of the CTX prophage is an integrated 4.7-kb plasmid, the toxin linked cryptic element (TLC), which was found in all CTX VPI *V. cholerae* isolates. Similarly, the class 1 integron which encodes antibiotic resistances was found in all toxigenic *V. cholerae* isolates. However the SXT element, a 62.5-kb conjugative transposon or constin that encodes several antibiotic resistance genes, was present in all serogroup O139 isolates but sporadic in occurrence among O1. This study demonstrates the co-occurrence of many of these mobile genetic elements within epidemic isolates of *V. cholerae* illustrating the role horizontal gene transfer has played in the emergence of pathogenic isolates.

MI 15 Withdrawn

MI 16 Do Shiga toxin-producing *Escherichia coli* cells survive Stx2 production?

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¹Depts of Microbiology and Immunology, University of Leicester, LE1 9HN, ²University of Newcastle, NE2 4HH Shiga toxins, key virulence factors produced by the emergent Shiga toxin producing *Escherichia coli* (STEC) group of pathogens, are encoded by the *stxA* and *stxB* genes which reside on the genomes of λ -like bacteriophages, downstream of the phage late promoter *p_R*. We and others have shown that phage induction, elicited by the SOS response, results in substantial increases in the transcription of *stx2* and the production of active toxin. However, phage induction also leads to cell lysis, and we have examined the implications of this with respect to the host bacterium, the phage and the toxin genes. In particular, the apparent inextricable linkage between *stx2* expression and lysis raises questions about the selection pressures that maintain the relationship between the phage and its bacterial host. We have addressed these issues by studying *stx2* expression at the single-cell level in an STEC strain carrying a chromosomal *stx2::lacZ* fusion. Cytological β -galactosidase assays have revealed that less than 20% of cells transcribe *stx2* in the absence of an SOS induction stimulus. Moreover, in these uninduced cultures *stx2::lacZ* expression appears to lead to a growth arrest and loss of cell envelope integrity. Our results indicate that expression of *stx2* is lethal to the host cell in all conditions tested to date.

MI 17 Horizontal transfer of mobile genetic elements encoding virulence genes in hospital strains of *Staphylococcus aureus*

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S. aureus is the most frequent cause of hospital acquired infection which is often difficult to treat due to antibiotic resistance. *S. aureus* carry multiple virulence genes on bacteriophages and mobile pathogenicity islands (SaPIs), including superantigen genes, leukocidins and other toxins. We investigated *S. aureus* strains in our hospital and showed they could be grouped into clonal clusters based on stable “core” gene restriction fragment length polymorphisms (RFLPs). When we looked at the carriage of “accessory” virulence genes in otherwise identical strains, we found evidence of frequent horizontal transfer of virulence genes. Furthermore, we isolated a pair of strains from one patient suggesting acquisition of a bacteriophage encoding the enterotoxin A gene *in vivo*. Our results demonstrate horizontal transfer, and perhaps loss, of mobile genetic elements occurs frequently in the hospital setting, and is likely to impact on the evolution of hospital adapted strains of *S. aureus*.

MI 18 The use of gene fusions to investigate differences in expression of locus of enterocyte effacement genes in human and bovine *Escherichia coli* O157

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Enterohaemorrhagic *Escherichia coli* (EHEC) are important causes of bacterial enteritis with the potential for progression to more serious syndromes. Many of the virulence factors involved in the development of the disease have been determined. These include the shiga-like toxins, secreted proteins (Esp) from the locus of enterocyte effacement (LEE) as well as other factors expressed from the 93 KB virulence plasmid, pO157.

Our previous data (McNally *et al.*, 2001) demonstrated that strains vary in their ability to produce the secreted proteins (EspD and Tir) the genes for which are located on LEE4. Following on from this work, the data presented will summarise our findings related to the expression and regulation of the promoter controlling LEE4 expression. We use stable, single-copy, translational fusions of the LEE4 promoter to *lacZ* and fluorescent protein reporter genes. Using different strain backgrounds, environmental conditions and cell lines, the factors regulating LEE4 are examined and will be reported.

MI 19 Regulation of expression of putative virulence factors located on the large plasmid pO157 of *Escherichia coli* O157 using promoter :: *lacZ* fusions

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Enterohaemorrhagic *Escherichia coli*, (EHEC) are a class of pathogenic *E. coli* that are associated with haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS) in humans. There are several different serotypes of EHEC, with *E. coli* O157 being the major pathogen in humans, particularly in the USA and UK. EHEC possess a 97kb plasmid, labelled pO157 in *E. coli* O157, which contains several genes implicated in virulence of the organism. These include an enterohaemolysin operon encoding an

RTX toxin, the *lif* gene encoding a large immunomodulatory molecule shown to inhibit lymphokine production, and *espP* which encodes a member of the SPATE family of exotoxins, protective antibodies against which have been found in convalescing human patients of O157 infection.

We have constructed promoter :: *lacZ* fusions, and using allelic exchange, have introduced these fusions into either *E. coli* MG1655 or *E. coli* O157 backgrounds in single copy into the native *lac* operon. This has allowed us to study environmental effects on the expression of *lif* and *espP*, and also for strain specific factors which may influence expression of these putative virulence factors, via β -galactosidase assays.

Initial results suggest that despite these two potential virulence factors being situated on the same virulence plasmid, their regulation differs with respect to environmental conditions.

MI 20 The epidemiology of the *rag* locus of *Porphyromonas gingivalis*

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Porphyromonas gingivalis is a Gram-negative anaerobic bacterium strongly associated with periodontal disease, a chronic inflammatory condition affecting the tooth supporting tissues. Certain strains of *P. gingivalis* harbour a novel pathogenicity island (PAI), the *rag* locus, which is correlated with increased virulence in both a murine model and in clinical samples by PCR. The locus consists of two genes *ragA* and *ragB* and is believed to have been acquired by *P. gingivalis* from another organism by horizontal gene transfer. In the present work we examined the chromosomal location of this locus and its heterogeneity in a collection of laboratory and clinical strains. Forty-four isolates were subject to strain typing by PFGE. Whole chromosomal DNA was digested with *Xba*I restriction enzyme and separated on a 1% CHEF agarose gel. The isolates were separated into 14 distinct PFGE types by their restriction profile. Southern blot hybridisation was used to investigate the chromosomal location of the *rag* locus. These analyses demonstrated that the locus is present at the same chromosomal location in all *rag* positive isolates, suggesting its acquisition by this species was a single historical event. Polymorphism in PAIs from other bacteria has been shown to affect virulence. To examine polymorphisms in the *rag* locus, long PCR was used to produce amplicons encompassing the entire *rag* locus of all positive isolates. The amplicons were digested with either *Bgl*II or *Sst*I restriction enzymes and the restriction profiles of the amplicons were compared to that of W50. The results showed that the locus is highly heterogeneous and that locus was polymorphic in isolates of the same PFGE type, suggesting that these polymorphisms may have occurred after acquisition of the locus. Heterogeneity exhibited by this locus may have important implications for the pathogenicity of *P. gingivalis*.

MI 21 A guinea pig virulence assay based on assessment of haematogenous dissemination

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To facilitate pathogenesis studies a guinea pig aerosol infection assay was developed to measure the virulence of strains of *M.*

tuberculosis. The assay was based on assessment of early dissemination of the bacilli from initial sites of infection in the lung to lymph nodes and spleen. This is a key feature of human infection that is replicated in guinea pigs.

M. tuberculosis strains Erdman, H37Rv and South Indian isolate TMC120 were used to aerosol infect groups of 8 guinea pigs with 10, 100 and 1000 bacilli (retained in the lung). Animals were killed at 16 days post infection and an infectivity index, defined as the dose required to cause a disseminated infection with 1000 bacilli in the spleen was determined. Groups of animals receiving 10 cfu were also killed after 8 weeks infection for histopathological analysis of lung sections.

The South Indian strain was significantly less virulent than Erdman or H37Rv with a higher dose (infectivity index) required to cause disseminated infection. Histopathology also confirmed that the South Indian strain was least pathogenic as a greater proportion of lesions were encapsulated and calcified; these are features of low pathogenicity which were present infrequently in H37Rv infected lungs. The infectivity indices for strains H37Rv and Erdman were comparable although histopathology indicated that Erdman caused more extensive consolidation and caseation of lung lesions. The infectivity index of strains was thus an indicator of pathogenicity and provided a means of comparing the virulence of strains. (*This work was supported by the Dept of Health, UK*)

MI 22 Genes encoding nucleotide cyclases and a cyclic nucleotide binding protein are upregulated during mycobacterial stationary phase

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During latent tuberculosis *Mycobacterium tuberculosis* bacilli persist in what is thought to be a non-replicating state, probably within granulomas where nutrients and oxygen are likely to be limited. Adaptation to the persistent state probably involves responses to environmental signals which alter gene expression in metabolic pathways required for long term survival. We have been studying a transcription factor belonging to the CRP/FNR family which appears to have a cyclic nucleotide (cNMP) binding domain. Unlike FNR, this protein does not appear to sense oxygen and the encoding gene is markedly upregulated during aerobically grown stationary phase cultures, suggesting that it may be responding to a stationary phase-associated stimulus other than O₂. Alternatively, it may respond to O₂, but only during stationary phase. A knockout construct has been made for allelic replacement and screening is underway to enable phenotypic studies of the mutant. The cNMP binding domain may bind cAMP or cGMP, which as a complex, may potentially regulate a variety of promoters. cNMPs, which are synthesized by adenylate/guanylate cyclases, are well recognised as second messengers. Also, work published 26 years ago showed an increase in bacterial cAMP levels in macrophages infected with mycobacteria suggesting cAMP may play a role in pathogenesis. We have studied several *M. tuberculosis* nucleotide cyclases at the transcriptional level and found that all are strongly upregulated during stationary phase. These findings have implications for signal transduction pathways which may control novel regulatory pathways associated with long term survival in the host.

MI 23 Characterisation of a *Mycobacterium tuberculosis* two component regulatory mutant GRETTA ROBERTS¹, JOANNA BETTS², DEBBIE SMITH³, NEIL STOKER³ & TANYA PARISH¹

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In order for *Mycobacterium tuberculosis* to successfully establish an infection it must be able to adapt to the different physiological environments encountered *in vivo*. Two component regulatory systems are one way in which bacteria can modify gene expression in response to changes in environmental conditions such as pH, oxygen concentration or nutrient availability. These systems consist of two proteins, a membrane spanning sensor and a DNA-binding response regulator. The sensor protein is a histidine kinase which transduces an environmental signal to the regulator by phosphorylation. The regulator then acts to either activate or repress other genes at the transcriptional level.

Eleven two component regulatory systems have been identified in the *M. tuberculosis* genome. We have constructed a mutant in the sensor protein of one of these systems (*senX3*). We have carried out phenotypic analysis of this mutant using both *in vitro* and *in vivo* models. No difference in growth kinetics, and ability to tolerate extreme pH were observed from the parental strain H37Rv. However, in a SCID mouse model of infection the mutant showed slight attenuation of virulence as compared to the parental strain. We prepared RNA from the mutant and wild-type strains and hybridised these to *M. tuberculosis* whole genome microarrays. Several genes appeared to be differentially expressed between mutant and parental strains. (*This work was funded by the GlaxoSmithKline Action TB Initiative*)

MI 24 Survival of *Mycobacterium* species in the Protozoan *Acanthamoeba castellanii*

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Bovine tuberculosis, caused by the intracellular pathogen *Mycobacterium bovis*, is of great economic importance to cattle farmers. Transmission is thought to be by deposition on pasture of bacteria in badger urine, faeces, pus or bronchial secretions. Due to the effects of ultraviolet radiation and desiccation, *M. bovis* bacilli cannot persist for an extended period in the environment. The potential for infection is therefore lessened. *M. bovis* may survive in the soil environment for longer if associations with the natural soil protozoan populations are formed.

We have studied the interaction between three mycobacteria, *M. bovis* BCG Pasteur, *M. bovis*, and *M. avium*, and the protozoan *Acanthamoeba castellanii*, at 15°C. The results showed substantial differences in the survival of the strains within the protozoan. While all strains were ingested by *A. castellanii*, *M. bovis* BCG Pasteur was unable to survive within the protozoan. In contrast, the pathogens *M. bovis* and *M. avium* showed continued survival. The protective effects conferred by survival of *M. bovis* within amoebae against UV radiation, pH, disinfectants and desiccation were studied. The results indicate that protozoal species may play a role in the survival of *M. bovis*

in the soil environment, and hence be involved in the transmission of bovine tuberculosis to cattle.

MI 25 The immunogenicity of a novel TB vaccine in guinea pigs - a comparison with BCG

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New TB vaccines are urgently needed if the global tuberculosis (TB) pandemic is to be controlled. In this study we tested a vaccine derived from a supernatant taken from a chemostat culture where *Mycobacterium tuberculosis*, H37Rv, had been grown under conditions relevant to an *in vivo* environment. In this pilot study we analysed various immunological parameters using the guinea pig model following vaccination. In pre-challenge animals we looked for the presence of TNF- α following the *in vitro* stimulation of splenocytes. We also set-up *in vitro* co-cultures and examined the ability of splenocytes, taken from vaccinated animals, to inhibit the growth of bacilli in infected macrophages following a 7-day incubation period. Finally, animals were challenged with aerosolised H37Rv. The number of bacteria detected in the lungs and spleens was used as a measure to determine protection. A comparison with BCG was also made.

MI 26 Definition of immunological correlates of pathology in cattle experimentally infected with *Mycobacterium bovis*

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Background: In Great Britain a scientific review has concluded that the development of a cattle vaccine against *Mycobacterium bovis* (*M. bovis*) infection holds the best long-term prospect for tuberculosis control in British herds. Vaccine development and the understanding of the pathology of bovine TB in cattle would be greatly facilitated by the definition of immunological correlates of protection and/or pathology.

Methods: Cattle were vaccinated with Bacille Calmette-Guerin (BCG) and then challenged with virulent *M. bovis*. Applying a scoring system of pathology, we were able to demonstrate that BCG vaccination reduced the disease severity by ca. 70 %.

Results: Analysis of cellular immune responses throughout the experiment indicated that proliferative T cell and IFN- γ responses towards the *M. bovis* specific antigen ESAT-6, whose gene is absent from BCG, were generally low in vaccinated animals, but high in all non-vaccinated calves. The amount of ESAT-6 specific IFN- γ measured by ELISA as early as 5 weeks after *M. bovis* challenge, and not the frequency of responding cells, correlated positively with the degree of pathology found 18 weeks after infection ($R^2 = 0.7972$, $p = 0.0005$).

Conclusion: These results suggest that the ESAT-6 specific IFN- γ is a useful prognostic immunological marker predicting both vaccine efficacy and disease severity.

MI 27 Developing novel vaccine candidates against *Mycobacterium bovis*: characterisation of an *M. bovis* BCG methionine auxotroph generated by transposon mutagenesis

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Background: One of the highest priorities in bovine tuberculosis research is to develop new vaccines for the long-term control of *Mycobacterium bovis* infection in cattle. One approach to the development of improved vaccines is to use a functional genomics approach to identify genes that are important for metabolism, intracellular survival and virulence of *M. bovis*. Transposon mutagenesis provides a direct selection for such mutants.

Methods: We have used a mycobacterial shuttle phasmid vector, pHA77, to produce a transposon library of *M. bovis* BCG. Southern blot analysis was used to confirm that transposition had occurred at random within the genome. The initial selection that we applied to the mutant library was to screen for mutants requiring specific amino acids, as such auxotrophs of other pathogenic bacteria have been shown to be attenuated. Ligation-mediated PCR (LM-PCR) was used to identify the transposon insertion site in selected auxotrophs.

Results: Screening 1500 mutants from the library isolated a *M. bovis* BCG mutant that required methionine for growth. The methionine auxotrophy was shown to be caused by disruption of *cysA*, encoding a component of the sulphate transport system. Biochemical studies clearly showed that the *cysA* mutant was defective in the transport of sulphur, while complementation with the wild-type *cysA* gene restored sulphur transport.

Conclusion: Investigations into the *in vivo* survival and global protein expression of this mutant are ongoing. Such analyses will show whether this strain has potential as a live attenuated vaccine, as well as offering insight into sulphur metabolism in tubercle bacilli.

MI 28 Putative virulence determinants of the Honey Bee pathogen *Paenibacillus larvae* subsp. *larvae* are present on a large defective phage particle

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Paenibacillus larvae subsp. *larvae* (*Pll*) is a Gram-positive rod shaped bacterium that is the causative agent of American Foulbrood of HoneyBees. *Pll* was compared to its non-virulent close relative *Paenibacillus larvae* subsp. *pulvificiens* via subtractive hybridisation. A unique 1.9 Kb fragment was identified, cloned, sequenced and analysed. Analysis of the sequence indicated homology with genes of unknown function within the *Lactococcus lactis* subsp. *cremoris* temperate bacteriophage ϕ 1t. The sequence was found to be present in a range of isolates including those thought to be "hyper-virulent". Furthermore, this sequence was used as a probe in order to clone a larger genomic fragment. Further sequencing revealed regions that shared homology with ClpP protease and the *yopB* component of the type III secretory-pathway of *Yersinia pestis*, indicating the presence of a pathogenicity island. Currently, this sequence is being further analysed in order to identify other virulence determinants. A model of the infection process of *Pll* encompassing these virulence determinants will be discussed.

WEDNESDAY 12 SEPTEMBER 2001

0910 Rerouting of carbon metabolism in *Lactococcus lactis* by metabolic engineering

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Lactic acid bacteria (LAB) are used world-wide in the industrial manufacturing of fermented food products. Their most important application in this respect is in dairy industry, where these micro-organisms are used to convert milk or milk-derived products to an enormous variety of fermented dairy products. The micro-organisms in milk are completely focussed on rapid conversion of the milk sugar, lactose, to lactic acid leading to acidified milk products. Beside this acidification that acts as a natural preservative, the LAB are important for the production of a variety of other metabolic end-products that contribute to desired product properties like flavour, shelf-life, and texture. The metabolic conversions that generate these end-products vary widely, depending on the lactic acid bacterium. Metabolic engineering strategies that involve inactivation of undesired genes and/or overexpression of existing or novel ones have been employed to create rerouting of the metabolic fluxes.

The major advantages of using LAB for metabolic engineering are their relatively simple metabolism, their genetic accessibility and the general lack of gene-multiplicity. Moreover, the extensive knowledge of metabolic pathways and the increasing information of the genes involved allows for the rerouting of natural metabolic pathways by genetic and physiological engineering. In this presentation several successful strategies to reach (efficient) rerouting of carbon metabolism in LAB will be discussed. Most research on lactic acid bacteria has been done with the cheese and buttermilk starter bacterium, *Lactococcus lactis*. The genetics and physiology of this lactic acid bacterium are well-known and a very efficient and tightly controlled (over)expression system is available, called the NICE-system. This system has been used successfully or is being used to induce in *L. lactis*, high production of the flavour compound diacetyl, the amino acid alanine, the structure components polysaccharides and the vitamin folic acid.

0950 Engineering bacteria for secondary metabolite production

I. HUNTER (University of Strathclyde)

Abstract not received

1100 Molecular breeding of novel carotenoid biosynthetic pathways

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Nature produces a stunning number of structurally diverse small molecules with different biological functions, making them highly valuable for the discovery of novel drugs and chemicals. Unfortunately, many of these compounds are only found in trace quantities in their natural sources and their

structural complexity makes chemical synthesis often difficult or impossible. Recombinant cells have been engineered for the production of complex natural products, but the number of compounds that can be produced in recombinant cells is small compared to natural diversity. More recently, combinatorial approaches involving recombination of biosynthetic enzymes and catalytic modules into new pathways emerged for the synthesis of additional complex metabolites. However, reliance on finding appropriate biosynthetic enzymes and catalytic modules that can possibly be combined into a functional pathway drastically limits the compounds that can be synthesized in engineered organisms.

The principles of breeding and *in vitro* evolution on the other hand can be used to access natural and non-natural product diversity rapidly and often in simple laboratory organisms such as *E. coli*. We combined techniques of metabolic engineering with those of molecular evolution to create novel carotenoid biosynthetic pathways in *E. coli*. Assembly of carotenoid biosynthetic genes from different organisms into pathways and *in vitro* evolution of key-enzymes within the context of the assembled pathways created novel biosynthetic routes for the synthesis of a number of acyclic and cyclic carotenoids in *E. coli*.

1140 Functional analysis of metabolic networks using elementary modes

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As genome sequencing gathers pace, some micro-organisms are being sequenced in the absence of any extensive biochemical knowledge, so we need to know whether an annotated sequence is a sufficient basis for inferring the metabolic capabilities of the organism. The metabolic potential of an organism cannot be expressed as a simple sum of those classical pathways for which it possesses the enzymes. The complement of enzymes expressed by an organism specifies a metabolic network, and we need to determine the characteristics of the routes available through this net, preferably by an algorithmic process. Elementary modes analysis is a method for finding all the feasible routes through such a network from nutrients to outputs. Its uses include computing theoretical maximum yields of products from given nutrients, along with related near-optimal routes. This will be illustrated for tryptophan synthesis pathway in *E. coli* and penicillin synthesis by *P. chrysogenum*. It can also determine the routes remaining after a particular enzyme has been deleted or inhibited, or equally whether new functioning routes will arise on expressing one or more additional enzymes in a transgenic organism.

1400 Engineered biosynthesis of nonribosomal peptides: application of molecular genetic and functional genomic technologies

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Nonribosomally synthesised peptides are a major class of commercially important microbial natural products. The peptide moiety of these products is synthesised on giant modular nonribosomal peptide synthetases (NRPSs) via the thiotemplate mechanism. Each 'module' specifies the incorporation of a

specific amino acid into the product and contains dedicated adenylation, condensation and thiolation 'domains'. Furthermore, the linear order of the modules within the giant polypeptides dictates the primary sequence of the peptide product. This colinearity provides great scope for 'reprogramming' NRPS-encoding genes to produce novel peptides. Engineered biosynthesis of NRPS-derived model peptides has been achieved with segments of NRPSs in *E. coli* and with model systems in *B. subtilis*. We are focussing on attempting to engineer NRPS pathways in the model actinomycete, *Streptomyces coelicolor*, using the gene cluster for the calcium-dependent antibiotic (CDA) a cyclic lipo(undeca)peptide. CDA bears similarity to daptomycin (Cidecin) derived from *Streptomyces roseosporus*. The latter is currently in phase III clinical trials as a potent antibiotic against vancomycin resistant Gram-positive isolates.

We are adopting a broad approach to manipulating the CDA biosynthetic pathway. This includes site-directed mutagenesis of amino acid specificity-conferring domains, module swapping, module addition, feeder pathway mutagenesis, mutasynthesis, and whole gene cluster up-regulation. It is clear that functional genomic technologies, such as DNA microarray analysis of the transcriptome will provide powerful new insights into the global consequences of manipulating individual metabolic pathways, or transcription factors. This technology is now emerging for *Streptomyces*. Current progress on the engineering of NRPS-derived products and the application of DNA microarrays in this area will be reviewed in this talk.

1440 Engineering *Deinococcus radiodurans* for environmental biotechnology: a tenacious bug with a taste for toxic waste

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Seventy million cubic meters of ground and three trillion liters of groundwater have been contaminated by leaking radioactive waste generated in the United States during the Cold War. A cleanup technology is being developed based on the extremely radiation resistant bacterium *Deinococcus radiodurans* that is being engineered to express bioremediating functions [1]. We have demonstrated that *D. radiodurans* can be genetically engineered for metal remediation and organic toxin degradation using expression systems that are functional during growth of the bacteria at 60 Gy/hour [2,3]. Recent progress in *D. radiodurans* research that is facilitating this work includes whole genome sequencing [4], genome annotation [5] and high throughput proteomics [6]. The *D. radiodurans* strain R1 genome (3.3 Mbp) consists of two chromosomes, one megaplasmid and one plasmid encoding 3,195 predicted genes. Sixty-one percent of the predicted proteome of *D. radiodurans* has been characterized with high confidence using a newly developed strategy based on accurate mass tags and represents the broadest proteome coverage for any organism to date [6].

With respect to U.S. Department of Energy (DOE) facilities, there has been no adequate method for microbiological treatment of contaminant waste sites containing both hazardous organic and radioactive metal components since organisms like *Pseudomonas* spp. are very radiation sensitive. High concentrations of toxic organic compounds (e.g., toluene) in mixed radioactive wastes pose as viable carbon/energy sources for metal-remediating

Deinococcus and could provide an effective alternative to conventional physicochemical treatments of radionuclides and toxic organic compounds. An assortment of organic toxin-degrading genes has been tested in *D. radiodurans* in combination with genes encoding resistance to Hg(II), Pb(II), and Cr(VI). In the presence of toluene, *D. radiodurans* expressing *todC1C2BA* produces toluene-*cis*-dihydrodiol that is further metabolized to 3-methylcatechol by a native non-specific dehydrogenase. Other *Pseudomonas* catabolic genes that convert 3-methylcatechol to pyruvate have been introduced into *todC1C2BA*-containing *D. radiodurans* yielding a strain that is able to mineralize toluene.

Substantially adding to the significance of our research on engineering *D. radiodurans* for bioremediation of DOE radioactive mixed waste sites are two recent developments 1) anaerobic cultures of wildtype *D. radiodurans* can reduce U(VI), Tc(VII) and Cr(VI); and 2) *Deinococcus* has been isolated from some of the most radioactive environmental samples ever collected at the Hanford (WA, USA) nuclear waste storage facilities (mainly contaminated with Cs, but also containing some Sr, U, and Tc: >10 E7 pCi/gram). Initial characterization of these isolates shows that they are closely related to and are equally resistant to ionizing radiation as *D. radiodurans* strain R1, and can also reduce Cr, U, and Tc.

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1550 Reprogramming bacterial terpene catabolism

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Biotransformations based on existing catabolic pathways are often constrained by the boundaries of evolutionary diversity rather than mechanistic constraints. However, protein and metabolic engineering now offer the prospect of constructing

metabolic sequences *de novo*. After a lack of success with traditional isolation and screening approaches we have recently started to explore the possibility of redirecting the bacterial catabolism of S(-)-pinene in *Pseudomonas fluorescens* NCIMB 11671 towards R(-) carvone production. This will involve constructing a 3 step pathway in which the second step of pinene catabolism, catalysed by pinene oxide lyase, is re-engineered to produce carveol.

Strategies to achieving these goals and preliminary results will be presented.

1630 After a decade of progress, an expanded role for Metabolic Engineering

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Metabolic engineering emerged as the field aiming at cell improvement using modern genetic tools. As such, it comprised a synthetic part of pathway engineering implemented by applied molecular biological methods and an analytical part for evaluating cell physiology using isotopic tracers, flux analysis, and computational methods. Numerous applications of metabolic engineering have appeared in the past decade and more are expected in future. An important characteristic of metabolic engineering is the emphasis it places on a *systemic approach* to cell physiology analysis and modification, in contrast to the single gene or enzyme focus of most reductionist approaches. This focus on *integration* uniquely qualifies metabolic engineering for the upgrade of the information content of the large volumes of data that will be generated by modern genomics-based methods. These data require a cell-wide framework for their analysis which is the central focus of metabolic engineering. In this talk we will propose that metabolic engineering can provide a most suitable vehicle for the analysis of expression, proteomic and metabolic data aiming at the integrated elucidation of metabolism, signal transduction and, in general, cellular function. Examples from metabolic and signaling pathways will be used in conjunction with expression data to illustrate a new, expanded role of metabolic engineering in industrial and medical applications and drug discovery.

POSTERS:

PBMG 01 Multiple DNA ligases in mycobacteria

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DNA ligases catalyse joining of DNA ends, a critical step in the replication, repair and recombination of DNA. As the first step in ligation, all DNA ligases form a covalent enzyme-adenylate intermediate, using either NAD⁺ or ATP as the donor of the adenylate group. The uniqueness of NAD⁺-dependent DNA ligases to eubacteria makes them an attractive target for novel antibiotics.

As of April 2001, 37 eubacterial genomes have been completely sequenced. Our analysis of these genomes shows that putative ATP-dependent ligases coexist with NAD⁺-dependent enzymes in 12 of these genomes, suggesting that a significant proportion of eubacteria may utilise both classes of enzymes. Currently, it is unknown why some eubacteria require both types

of DNA ligase. Remarkably, *Mycobacterium tuberculosis* contains genes that are predicted to encode one NAD⁺- and three ATP-dependent DNA ligases. *M. leprae* also contains homologous sequences, but – like many sequences in this organism – the three ATP-dependent DNA ligases are pseudogenes. These observations question whether these putative genes for ATP-dependent DNA ligases play any functional role(s) in the cell.

If DNA ligases are to be targeted effectively by novel antibiotics, it is important to identify the role of different DNA ligases within the same bacterium. To begin to address this question, we have cloned and over-expressed DNA ligases from *M. tuberculosis*. Purification and biochemical analysis of these proteins is in progress.

PBMG 02 Rapid extraction of genomic DNA from *Mycobacterium tuberculosis* for use in Southern hybridisation experiments

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The construction of null mutants is a valuable tool in the study of bacterial gene function. For *Mycobacterium tuberculosis* (M.tb), creation of such mutants in targeted genes is often hampered by the high proportion of illegitimate recombination events that take place. Typically, many putative mutants will have to be screened before a legitimate allelic exchange mutant may be identified. PCR may not always give clear-cut evidence of legitimate allelic exchange, thus the ‘gold standard’ for mutant characterisation lies in Southern hybridisation analysis of extracted chromosomal DNA.

Genomic DNA extraction from M.tb is made difficult and time-consuming by the thick, lipid membranes of the bacilli, which render the organism resistant to chemical and enzymatic attack. Mechanical lysis of the cells may shear the genomic DNA, such that Southern hybridisation analysis becomes difficult. We have applied a simple, fast and effective method for the extraction of genomic DNA from M.tb by mechanical lysis using a Ribolyser, and have found that the DNA obtained is suitable for Southern hybridisation analysis of fragments of up to 11kb. Many DNA extractions may be performed concurrently, allowing a high throughput of mutant analyses.

We describe the use of this method in the identification of a single *mce2Δ::kan* mutant from a pool of 50 potential mutants.

PBMG 03 Growth stimulation of non-culturable *Mycobacterium bovis* (BCG) cells by Rpf-like proteins

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Prolonged incubation of *Micrococcus luteus* cells in stationary phase causes the production of nonculturable cells with a low level of metabolic activity. These cells can be resuscitated by the addition of a protein (Rpf - Resuscitation promoting factor) secreted by actively growing micrococci. Genome sequencing projects have revealed that other members of the high G+C cohort of Gram-positive bacteria contain genes whose products resemble Rpf. There are five such genes in *Mycobacterium bovis* & *Mycobacterium tuberculosis*.

We therefore determined whether (a) *Mycobacterium bovis* (BCG) can produce non-culturable forms similar to those of *M. luteus* and (b) they can be resuscitated by a selection of recombinant Rpf-like proteins. During prolonged incubation in stationary phase, BCG cultures in Sauton's medium gradually produce non-culturable forms with low metabolic activity. A culture 45 d post-inoculation had a total count of 1.4×10^9 cells/ml with approximately equivalent CFU and MPN values (8.1×10^8 & 1.6×10^9 cells/ml, respectively). In contrast, bacteria 5 mo. post-inoculation had completely lost culturability. The total count was 7.7×10^8 cells/ml, with CFU and MPN values of <5 & <10 cells/ml, respectively. Significantly, the addition of a recombinant form of *M. luteus* Rpf restored culturability, giving an MPN value of 4.8×10^8 cells/ml.

Recombinant forms of any of the five Rpf-like proteins of *Mb. tuberculosis* also stimulated the growth of non-culturable cells of BCG at picomolar concentrations. Moreover, experiments with a truncated form of Rpf revealed that activity resides in the 70-amino acid "Rpf domain" shared by all Rpf-like proteins. Antibodies raised against Rpf completely inhibited the growth of BCG using an inoculum of cells in early stationary phase, showing the importance of Rpf for recovery of these bacteria.

These experiments show that BCG can produce nonculturable but viable forms in laboratory culture. Growth of these cells is absolutely dependent on addition of an Rpf-like protein. These findings have important implications for the study of latent mycobacterial infections and may contribute to a future strategy permitting their eradication.

PBMG 04 *glnE*: an essential gene of *Mycobacterium tuberculosis*

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Interconversion of glutamate and glutamine is a key reaction in maintenance of the nitrogen balance in bacterial cells. In addition, *Mycobacterium tuberculosis* is unusual in synthesizing extracellular poly-L-glutamine, and in containing D-iso-glutamate in the peptidoglycan. *M. tuberculosis* contains 4 glutamine synthetases (GSs), one of which has been shown to be essential. While constructing auxotrophs of this pathogen, we were unable to isolate a mutant in *glnE*, the adenylase that regulates activity of Type I GSs. We have constructed a strain containing a functional copy of *glnE* on a temperature sensitive vector and an inactivated chromosomal copy (Glue9). We have monitored plasmid loss from Glue9 in liquid media and show that, as expected, the plasmid is not lost at 37°C. One possibility for the inability to isolate a *glnE* mutant is that the media we use is nitrogen-rich. We are currently exploring the possibility that *glnE* mutants are viable in limiting-nitrogen conditions. We have developed a synthetic medium in which we can easily change the nitrogen source, and tested various formulations for the ability to support growth of the wild-type strain. This media is being used to isolate a plasmid-free *glnE* knockout mutant from Glue9 at 37°C. So far, none has been isolated, but further media modifications are being tested.

PBMG 05 Formation and resuscitation of "non-culturable" mycobacteria in stationary phase

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The aim of this study was to find conditions for the transition of *M. phlei* and *M. tuberculosis* (MTB) to an ostensibly (i.e. immediately) "nonculturable" state and to test the hypothesis that the secreted bacterial cytokine Rpf (from *Micrococcus luteus*) may stimulate resuscitation of mycobacterial cells.

To establish an apparently "nonculturable" state cells were held in stationary phase at 37°C. Cell viability was determined by plating or by a most probable number (MPN) assay in the presence of supernatant (SN) taken from the culture of logarithmically growing cells or of added recombinant Rpf in the assay medium.

After incubation of *M. phlei* for 86-96 h and of *M.tuberculosis* for 4 months in stationary phase *in vitro*, there was a significant (up to 5 orders) decrease of the cfu count, while the total count was similar to its initial value. The MPN assay revealed that the viability was up to 100 times (for *M.phlei*) and 10^5 times (MTB) higher than that of the cfu count, and the presence of SN or picomolar concentrations of recombinant Rpf had an additional (1- 2 logs) beneficial effect on the viability by MPN. The best results (i.e. when the viable count was closest to the total bacterial count) were obtained for cultivation of "non-culturable" cells in liquid medium in the presence of SN.

PBMG 06 Genetic and structural studies of inositol metabolism in mycobacteria

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Inositol is used by mycobacteria in phosphatidylinositol (PtdIns), which anchors lipoarabinomannan in the cell wall, and in the major thiol, mycothiol, which helps maintain the redox balance of the cell. We have been studying genes involved in the early stages of inositol metabolism, notably inositol monophosphate phosphatase (IMPase) and inositol phosphate synthase (Ino1) genes.

Ino1 catalyses the conversion of glucose-6-phosphate to inositol-1-phosphate, while IMPases catalyse the production of inositol from inositol phosphate. There is one *ino1* gene, and three genes encoding IMPase homologues in *M. tuberculosis* (*impA-C*), all of which map to different parts of the genome. *M. smegmatis* has orthologues of these three genes, plus one additional homologue (*impD*). We have been studying the functions of these genes using genetic, biochemical and structural

studies. PtdIns dimannoside (PIM₂) is present at reduced levels in an *M. smegmatis impA* mutant, but mycothiol levels are normal. In contrast, PIM₂ (and mycothiol) levels are normal in an *M. tuberculosis impA* mutant, while in *M. leprae*, *impA* is a pseudogene. We have expressed, crystallised and determined the structure of *M. smegmatis impA*, and are modelling the homologues from this in order to carry out structure-function studies, and to compare these proteins with mammalian IMPases. (This work was funded by the Wellcome Trust, the ICRF and the BBSRC)

PBMG 07 Characterisation of a the *narL* response regulator gene of *Mycobacterium tuberculosis* H37Rv

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Mycobacterium tuberculosis has to withstand a number of hostile environments if it is to survive in the host. One of the mechanisms by which the bacterium senses environmental signals and responds accordingly is through two-component regulator (2CR) systems. In other pathogens such as *Salmonella typhimurium*, these systems are of central importance to bacterial survival and virulence. *M. tuberculosis* has 11 probable 2CR systems, and we have been systematically inactivating these in order to determine which of those are important in infection. Here we describe the inactivation and characterization of the response regulator gene *narL*.

PBMG 08 Cytochromes P450 as novel anti-tuberculosis drug targets

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Cytochromes P450 (P450s) are a superfamily of haem-containing enzymes that catalyse the activation and insertion of a single oxygen atom into a wide variety of substrates. Mammals and other eukaryotes have multiple P450s, involved in both metabolism and xenobiotic detoxification. Generally, bacteria have fewer P450s. However, *E. coli* has none. The genome sequence of *M. tuberculosis* indicated the presence of 20 P450s, the largest number found in a bacterial genome. Mycobacteria contain sterol biosynthetic pathways and, given the extremely unusual composition of their envelope, the P450s are likely to be involved in synthesis and inter-conversions of membrane lipids and sterols. Potent azole inhibitors already exist for numerous sterol metabolising P450s forming the basis of a billion dollar industry of anti-fungal agents. A number of *M. tuberculosis* P450s and their redox partners have been cloned and overexpressed. So far expression of P450 MT-2 (product of gene Rv2276) has been optimised to produce haem-containing, soluble P450 protein, which has been purified and characterised. MT-2 binds extremely tightly to a variety of azole drugs. These azoles are powerful inhibitors of growth of *M. paratuberculosis* and *Streptomyces coelicolor*. The latter bacterium (like *M. tuberculosis*) encodes a large number of P450s in its genome, many of which are homologous to those in *M. tuberculosis*. Inhibition of the *M. tuberculosis* P450s presents a new strategy to combat multi-drug resistant strains of the pathogen. The atomic structure of the *M. tuberculosis* P450 MT-2 has been resolved at 1.6Å, the first

P450 structure to be determined in Europe. In this high quality structure, 393 of the 396 amino acids are resolved. Molecular modelling has highlighted a number of residues that may be involved in binding of the azoles. Here we present the structure of our P450 MT-2 and its potential as a new anti-tuberculosis drug target.

PBMG 09 Characterization of cytochromes P450 in *M. tuberculosis* and *S. coelicolor*

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The genome sequence of *Mycobacterium tuberculosis* revealed the presence of an unprecedented number of cytochrome P450 enzymes for a bacterium. Up to 20 P450s are encoded in a genome of size similar to that of *E. coli*. By contrast, *E. coli* encodes no P450s. A high proportion of the *M. tuberculosis* proteome is involved in lipid metabolism, and most of the mono-oxygenase P450 enzymes are likely to be involved in metabolism of lipids, sterols and polyketides in the pathogen. More recently, the *Streptomyces coelicolor* genome sequencing project has shown that this organism also contains a plethora of P450-encoding genes, and that there are multiple homologues of the *M. tuberculosis* P450s in *S. coelicolor*.

Several of the genes encoding *M. tuberculosis* P450s and their redox partners (ferredoxin reductase and ferredoxin) have been cloned in our laboratory. These include the P450 encoded by the Rv0764c gene, which encodes the homologue of the eukaryotic sterol demethylase CYP 51 P450s. The fungal forms of this enzyme are targets for azole drugs that inhibit the formation of ergosterol, a key component of the fungal membrane. Here we report the cloning and characterization of *M. tuberculosis* and *S. coelicolor* P450s, including the sterol demethylase enzyme from *S. coelicolor*.

PBMG 10 Generation of mycobacterial mutants using plasmid incompatibility

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Efficient delivery systems are essential genetic tools for targeted gene replacement and transposon mutagenesis. Such delivery systems rely on introduction of DNA followed by vector loss. This has been accomplished in mycobacteria by the use of a suicide vector, a temperature sensitive replicon, or a conditional phage. We have been developing an alternative delivery system based on the use of replicating plasmids, which are then lost due to plasmid incompatibility. Plasmid incompatibility is the inability of a pair of co-resident plasmids to be stably maintained where they share a common replication control or segregation mechanism. We have shown that pAL5000 can be used efficiently to generate mutants in *Mycobacterium smegmatis*

using incompatibility to generate plasmid loss. The system was improved by separating the two replication genes, so that each plasmid is dependent on the other, and by introducing a second incompatibility region into one of the plasmids. We are increasing the flexibility of the plasmids for cloning purposes and are in the process of testing the system in *Mycobacterium tuberculosis*. (This work is funded by GlaxoWellcome Research & Development.)

PBMG 11 A DNA fragment from *Mycobacterium bovis* (BCG) activates secondary metabolism in *Streptomyces*

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We describe the isolation of a DNA fragment from a *Mycobacterium bovis* (BCG) genomic library capable of activating actinorhodin biosynthesis in *Streptomyces coelicolor* ATCC 10147 and *Streptomyces lividans* TK 54.

About 1000 thiostreptone resistant colonies of *S. lividans* TK 54 were obtained when transforming with the ligation mixture. One clone showed an intense blue pigmentation, typical of actinorhodin production. Plasmid DNA from the selected clone was purified and transformed into *S. lividans* TK 21, where the original activated phenotype was obtained, confirming that activation was plasmid borne.

Transformation of *S. coelicolor* JF1 and J1703 showed no complementation of the actinorhodin production deficiencies in these strains. Therefore the cloned sequence exerts its regulation by a mechanism different from that involving *actII* and *bld A* in the parental strain of *S. coelicolor*.

The above experiments established the existence of *M. bovis* BCG DNA fragment able to active the synthesis of secondary metabolites in *S. lividans* and *S. coelicolor*. This support previous claims that *Streptomyces* is a good host for cloning genes from slow growing mycobacteria.

The nucleotide sequence of a *M. bovis* BCG DNA fragment that activated antibiotic production in *Streptomyces* was determined. Remarkably high identities were found with DNA sequences from *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

PBMG 12 Isocitrate lyase from *Streptomyces coelicolor*: enzyme purified, gene cloned and sequenced - but still no 'hit' on the Sanger Database

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The glyoxylate bypass is a metabolic pathway used by many bacteria to replenish carbon that is 'drained off' from the TCA cycle for the biosynthesis of cellular monomers, and is particularly important to bacteria that are metabolising nutrients that are broken down directly to acetate (e.g. fatty acids). The carbon may flow **EITHER** round the TCA cycle via isocitrate dehydrogenase (Idh) **OR** through the glyoxylate bypass via isocitrate lyase (Icl). We have focussed on these two enzymes to

investigate the control of carbon flow at this branchpoint and to attempt to subvert this regulation in recombinant organisms.

The *icl* gene has been cloned from genomic DNA of *S. coelicolor*, by a 'reverse genetics' and sequence analysis has identified a 426 aa Orf that has high similarity to Icl sequences on the databases. When this *icl* clone was used to probe the original Redenbach cosmid library, nothing lit up. We have continued to scan the output from the *S. coelicolor* sequencing project at Sanger, but no 'hit' against *icl* has appeared.

Our *icl* clone contains a malate synthase (MS) gene that is adjacent, and presumably coordinately regulated. The order of these genes, (Icl proximal to MS), is unusual for bacteria. Using our malate synthase Orf to probe the Sanger database, cosmid SCAH10 is shown to have an orf that has high similarity and is >60% similar to *E. coli* AceB (one of two forms of malate synthase present in *E. coli*). We therefore conclude that at least two malate synthase genes are likely present in the genome of *S. coelicolor*.

PBMG 13 Microarray analysis of an *M. tuberculosis* *trcS* mutant: statistical modelling of replicates

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Microarray analysis is a powerful technique for the identification of differentially expressed genes in mutant organisms. In order to identify genes under direct or indirect control of the *trcRS* two-component regulatory system of *Mycobacterium tuberculosis*, cDNA from a *trcS* mutant was hybridized to glass-slide microarrays containing PCR products from all 3924 genes of *M. tuberculosis*. In order to develop statistical methods for identifying expression differences, six replicates of the same hybridization experiment were carried out. An analysis of variance was applied to reveal the amount of variation due to the various sources of experimental error. We fitted linear models, taking various combinations of these factors into account, and compared their explanatory power. Bootstrap resampling of the residuals of the best models was used to obtain p-values for the significance of expression levels of differentially expressed genes. In this way, we found about 10 genes with significantly different expression levels. Real-time PCR is being used to confirm these results.

PBMG 14 Mycolic acid biosynthesis and enzymatic characterization of the KasA condensing enzyme from *Mycobacterium tuberculosis*

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Mycolic acids are key components of the mycobacterial cell wall. In view of their importance in bacterial survival and virulence, enzymes involved in their metabolism represent attractive targets for the design of new anti-mycobacterial agents. Mycolic acids consist of long-chain ω -alkyl- ω -hydroxy fatty acids that are produced by successive rounds of elongation catalyzed by a type II fatty acid synthase (FAS-II). A key feature in the elongation process is the condensation of a two-carbon unit from malonyl-acyl carrier protein (ACP) to a growing acyl-ACP chain catalyzed by ω -ketoacyl-ACP synthases, which in *Mycobacterium tuberculosis* consist of KasA and KasB. Here, we provide evidence that *kasA* encodes an enzyme that elongates the meromycolate chain *in vivo*, in both *M. smegmatis* and *M. chelonae*. We also demonstrate *in vitro* that KasA belongs to the type FAS-II system which utilizes primarily, palmitoyl-ACP rather than short chain ACP-primers. Furthermore, highly purified recombinant KasA exhibited ω -ketoacyl-ACP synthase activity *in vitro*, in the presence of palmitoyl-AcpM and malonyl-ACP in a condensation assay. In addition, purified KasA was sensitive to thiolactomycin and cerulenin, two known inhibitors of ω -ketoacyl-ACP synthases. The data presented is consistent with a critical role for KasA in the mycolic acid biosynthetic pathway in mycobacteria and opens new avenues for the development of novel anti-mycobacterial drugs targeted against KasA.

PBMG 15 Transmission and Scanning Electron Microscopy Studies into the Lethal Effects of High Intensity Pulsed Electric Fields on *Mycobacterium paratuberculosis*

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Mycobacterium paratuberculosis is a thermotolerant acid-fast bacterium that has been associated with Johne's disease in cattle. It has been isolated recently from pasteurised retail-milk supplies in the UK. *M. paratuberculosis* has been suggested as a possible cause of Chron's disease in humans. Previous studies have shown that this microbial pathogen is only reduced by approximately 2.5 log orders in milk by pasteurisation.

Here, we report that treatment of bovine and human strains of *M. paratuberculosis* in bovine-milk with pulsed electric fields (PEF; 25 kV/cm) at 50°C reduced cell numbers by 6 log orders. Assessment of microbial inactivation occurred by plate count (CFU/ml) and by electron microscopy (EM). Transmission and scanning EM studies of PEF-treated *M. paratuberculosis* cells revealed irreversible membrane damage at the cellular level.

The inclusion of PEF, as a complementary inactivation technology to thermal pasteurisation, may afford additional protection against the possible contamination of retail-milk supplies by *M. paratuberculosis*.

SYSTEMATICS & EVOLUTION GROUP

Classification and identification of clinically significant actinomycetes

WEDNESDAY 12 SEPTEMBER 2001

0910 Epidemiology of aerobic actinomycete infections: an overview

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Most clinical aerobic actinomycete infections are caused by *N. asteroides* complex, *N. brasiliensis*, and *N. otitidiscaviarum*.

Novel pathogenic *Nocardia* species include *N. abscessus*, *N. africana*, *N. brevicatena*, *N. farcinica*, *N. nova*, *N. paucivorans*, *N. pseudobrasiliensis*, *N. transvalensis*, and *N. veterana*. Reports from reference laboratories in Australia, France, Germany, Italy, United Kingdom and the United States, have each demonstrated a recent increase in the number of *Nocardia* spp. isolates and emphasized the emergence of invasive infections attributed to *N. farcinica*. Infections with *Nocardia* spp. are usually sporadic; invasive pulmonary and disseminated disease occurs predominantly in severely immunocompromised patients, including HIV-infected patients. In addition, *N. farcinica* has recently been reported to cause nosocomial outbreaks. Aerobic actinomycetes well known to cause mycetoma in developing countries include *N. brasiliensis* and the genera *Actinomadura* and *Streptomyces*. Recently, other aerobic actinomycete genera identified to cause systemic infections in U.S. patients include *Gordonia*, *Oerskovia*, *Rhodococcus*, and *Tsakamurella*. The diagnosis of invasive aerobic actinomycete infections has been hindered by a combination of clinical and microbiologic difficulties, including their often nonspecific clinical presentation, a frequent requirement for invasive diagnostic biopsy procedures, difficulty in isolating the microorganism, and the imperfect classification of these related genera. Effective antimicrobial therapy for these infections may be complicated by: adverse effects of antimicrobial agents, antimicrobial resistance, and a lack of effective, antimicrobial agents. Optimal therapy may be guided by antimicrobial susceptibility testing of isolates.

0950 Actinomycetoma in Sudan caused by new organisms

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Actinomycetoma is a chronic, specific, granulomatous, progressive inflammatory disease. It usually involves the subcutaneous tissue, then it spreads to involve the skin and the deep structures resulting in destruction, deformity and loss of function, very occasionally it could be fatal.

Actinomycetoma has a worldwide distribution but this is extremely uneven. It is endemic in many tropical and subtropical regions. It prevails in what is known as the mycetoma belt stretching between the latitudes 15° south and 30° north. The belt includes Sudan, Somalia, Senegal, India, Yemen, Mexico, Venezuela, Columbia, Argentina and others.

Actinomycetoma is caused by higher bacteria (actinomycetes), and various types of actinomycetes were reported to produce the disease. In the Sudan, actinomycetoma is commonly caused by *Streptomyces somaliensis*, *Actinomadura madurae*, *Actinomadura pelletieri* and *Nocardia* spp. However, new organism has recently been reported from Khartoum, Sudan; it is clinically similar to *S. somaliensis*. The geographical distribution of the individual actinomycetoma organism shows considerable variations, which can be convincingly explained on an environmental basis. Areas where mycetoma prevails are relatively arid zones with a short rainy season with a relative humidity. The organisms are usually present in the soil in the form of grains. The infecting agent is implanted into the host tissue through a breach in the skin produced by trauma.

A painless subcutaneous mass, sinuses and purulent and seropurulent discharge containing grains characterize actinomycetoma. Clinically it is similar to eumycetoma but it is more aggressive, destructive and inflammatory than eumycetoma.

The commonest site for actinomycetoma is the foot. Extra-pedal disease is common and this more prevalent than that seen in eumycetoma. The disease is usually localized but the organisms may spread along the fascial planes to involve other structures. In about 1-3% of cases there is a genuine lymphatic spread to the regional lymph nodes. During the active phase of the disease these regional lymphatic foci may suppurate and discharge as well. The differential diagnosis of actinomycetoma includes many of the soft tissue granulomas and tumours.

Actinomycetoma can be diagnosed by radiology; it demonstrates the presence and the extent of the soft tissue and bone involvement. It may be of a prognostic value and for follow up of patients during medical treatment. The diagnostic cornerstone of actinomycetoma is the identification of the organism by culture; the organisms can be identified by their textural description, morphological and biological activities. Stained histological sections usually show the grain morphology and the tissue reaction to the organisms. The technique is attractive in that it requires neither aseptic procedure nor the rigid time schedule required for culture, however it lacks the precision of culture. Serodiagnosis is of a great help in identification and classification of the various organisms, which is an essential prerequisite for medical treatment, and is mandatory for the follow-up of these patients. It has many advantages over the culture and histopathological techniques, as both require surgical biopsy, which may enhance the spread of the organism. The common serodiagnostic tests for mycetoma are the immunodiffusion and counter-immuno-electrophoresis but cross reactivity between the individual organisms is quite common. Other diagnostic tools included, ultrasound and cytology both of them are simple, rapid and non-invasive.

Actinomycetoma is amenable to medical treatment with antibiotics and other chemotherapeutic agents. Combined drug therapy is always preferred to a single drug to avoid drug resistance and to eradicate residual infection. The common drugs in use include combination of streptomycin sulphate (14 mg/kg daily), diaminodiphenyl sulphone (dapsone) (1.5 mg/kg twice daily). If there is no response for few months or if there is persistent side effect then dapsone is replaced by Co-trimoxazole (14 mg/kg twice daily). An excellent therapeutic response to amikacin sulphate alone or in combination with Co-trimoxazole has been reported in the treatment of actinomycetoma.

1100 *Nocardia* spp. as pathogens of the brain: Is there an association with Parkinson's disease?

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Experimentally, some strains of nocardiae (neuroinvasive strains) adhere to capillary endothelial cells in the brain when log phase nocardial cells become blood borne. These adherent bacteria invade through the capillary and enter perivascular cells in the brain parenchyma where they may be observed within neurons, axonal extensions, nerve synapses, and both astroglia, and microglia. Electron microscopic observations reveal that the perivascular growth of these bacteria induces alterations in the integrity of the myelin sheath, alteration of nerve synaptic vesicles, and cellular degenerative changes. Even though there are numerous areas with nocardial invasion without an apparent host inflammatory response, in some brain regions there is cellular damage concomitant with infiltration by PMNs and mononuclear cells. *Nocardia asteroides* GUH-2 invades cryptically the *substantia nigra pars compacta* region (SNpc) in the brain in both

mice and monkeys. These experimental infections induce neurodegenerative and neurochemical changes that share features with Parkinson's disease (PD). Furthermore, molecular techniques suggest the presence of nocardiae in the SNpc in some cases of PD. Thus, there is significant data supporting the hypothesis that *Nocardia* species may be etiologically associated with some cases of PD, but to our knowledge, there are no data indicating that this hypothesis cannot be sustained.

1140 Lipoarabinomannan lipoglycans in mycolic acid-containing actinomycetes: a chemotaxonomic perspective

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The cell envelopes of actinomycete bacteria typically contain lipoglycans which are presumed to play crucial although as yet unknown physiological roles. Although these lipid-anchored polysaccharides are structurally diverse, the members of closely related taxa exhibit similarities in lipoglycan composition and thus lipoglycan distribution can be of chemotaxonomic value. We have been investigating lipoglycan diversity within the mycolic acid-containing actinomycetes (the mycolata). The archetypal lipoglycans for this taxon are the extensively studied lipoarabinomannans (LAM) of the genus *Mycobacterium* which exhibit considerable structural variation based around a common core structure consisting of a phosphatidylinositol anchor which bears a branched mannan and arabinan heteropolysaccharide. Numerous studies have documented the presence of 'LAM-like' lipoglycans with similar carbohydrate compositions in related members of the mycolata. This presentation will review recent progress in the characterisation of these LAM-like lipoglycans with an emphasis on variations on the LAM archetype. For example, the important equine pathogen *Rhodococcus equi* produces a distinctive variant in which the key structural features are a linear $\alpha(1-6)$ mannan backbone linked to a phosphatidylinositol anchor and carrying 2-linked mannose residues. In contrast to mycobacterial LAM there are no extensive arabinan domains but rather the mannan core is decorated with single arabinose residues. The broader chemotaxonomic significance of lipoglycan analysis will also be considered.

1220 The respiratory pathogen *Rhodococcus equi*: steps towards some taxonomic solutions

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The respiratory pathogenic actinomycete *R. equi* was assigned to the genus *Rhodococcus* in 1977, but despite the fact that the intervening period has seen significant advances in the systematics of related microbes, the status of the *R. equi* taxon is yet to be clearly established. The genus *Rhodococcus* is both heterogeneous and paraphyletic. Phylogenetic analysis of 16S rDNA sequence data suggests that *R. equi* is more closely related to organisms in the genus *Nocardia*. Although 16S rDNA sequencing can rapidly establish evolutionary relationships, it is essential that they are validated using other taxonomic criteria

including phenotypic characterisation, in a polyphasic taxonomic approach.

Phenetic data were amassed on the large collection of *R. equi*. This collection included fresh isolates from the environment as well as strains from humans and other animals plus strains from culture collections along with type strains of other rhodococcal and nocardial species. Rapid phenotypic data acquisition was facilitated using the BiOLOG GP2 Microplate™ system and pyrolysis mass spectrometry. A classical numerical phenetic taxonomic study was also carried out and all analyses of phenotypic data compared with the 16S rDNA phylogeny of *R. equi*.

Keywords: *Rhodococcus equi*, polyphasic taxonomy, BiOLOG, pyrolysis mass spectrometry, 16S rDNA sequencing, numerical taxonomy

1400 The Natural History of *Actinomyces*

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Actinomyces are consistently present within the resident oral flora of humans and other animals. It is less certain that they inhabit other body sites, although they are isolated from tonsils. *Actinomyces* spp. colonize the oral cavity of infants soon after birth and at tooth eruption they contribute to biofilm formation on tooth enamel. *A. naeslundii* fimbriae facilitate adherence and also mediate co-aggregation and co-adherence among oral bacteria, both significant processes in dental plaque formation. Currently, the genus includes 21 species, some which have genetic variants. Many ribotypes of *A. naeslundii* exist and up to 20 ribotypes can colonize a human. *Actinomyces* occur in mixed infection abscesses e.g. *A. israelii* and *A. gerencseriae* contribute to actinomycosis. Also, newly recognized *Actinomyces* have been isolated from blood and other sites, however, the aetiology of these infections is not known. The biofilm habitat and genetic and phenotypic diversity of *Actinomyces* can be suggested to contribute to the survival of species. Studies using a biofilm model inoculated with 5 ribotypes of *A. naeslundii* have shown that one ribotype may represent the species in a given environment but the other ribotypes remain in the biofilm. Environmental change causes a second ribotype to dominate; however the previously dominant ribotype survives in the biofilm.

1440 Immunodiagnosis of infections due to *Nocardia*: what's new?

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The first immunological test for diagnosis of nocardiosis is based on the detection of the 54/55-kDa protein of *Nocardia*. Although this antigen is easily detected in pulmonary and systemic nocardiosis, it is rarely detected in cutaneous nocardiosis.

A murine model was used to develop a sensitive and serologic test for infection caused by *Nocardiae*. Extracellular proteins of 22 kDa, 24 kDa, 31 kDa, 36 kDa, 43 kDa, 62 kDa and 90 kDa have been identified as possible antigens. They could be used as additional markers for immunodiagnosis and identification of pathogenic *Nocardiae*.

1600 Novel approaches for the identification of Nocardiae

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The genus *Nocardia* has had a long and tortuous taxonomic history mainly due to an overemphasis placed on morphological criteria. This state of chaos ended after the application of chemical and molecular systematic methods, notably 16S rDNA sequencing. The revised classification provides a sound framework for the recognition of additional species though improved methods are needed, notably for the rapid and reliable assignment of unknown clinically significant nocardiae to validly described and putatively novel species. In the present study, a fragment of the *rpoB* gene (ca. 2000 nt) was sequenced and the resultant phylogenetic tree compared with that obtained from 16S rDNA gene sequencing data. A further analysis of the amplified region was performed in the search of a molecular marker for the reliable identification of clinically significant nocardiae. It is evident that this small region of the *rpoB* gene provides a suitable target for the improvement of current methodologies employed in nocardial identification. The identification of clinically significant nocardiae to the species level is critical for establishing the spectrum of disease produced by members of each species and for predicting antimicrobial susceptibility.

1630 Fast-growing mycobacteria: an emerging clinical problem

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There are, at present, 92 validly described species and subspecies encompassed by the genus *Mycobacterium*, of which 50 are considered pathogens of animals or man. It is usually accepted that the majority of pathogenic isolates will be assigned to one of the slowly-growing species. However, there are now 15 fast-growing mycobacterial species described as pathogenic, of which 11 are opportunistic human pathogens. New developments in health-care have led to a re-appraisal of the interaction between fast-growing environmental mycobacteria and man. Such developments principally involve invasive procedures, such as endoscopy and haemodialysis, and immunosuppressive disorders and therapies, such as fibrocystic disease. Among those organisms detected in samples from patients undergoing such procedures, mycobacteria, which commonly occur in aquatic environments, are of increasing concern. Given that exposure to environmental mycobacteria may often be beneficial, the characteristics of those species likely to be a threat to vulnerable individuals need clear definition and a better understanding of the taxonomy of these organisms is needed.

POSTERS:

SE 01 Unravelling the mycobacterium chelonae complex

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Members of the *Mycobacterium chelonae* complex are widely distributed in the environment, notably in aquatic habitats. They are common in natural water systems but are not eradicated by waste water treatment processes. Members of the complex present few problems for healthy individuals but are an increasing risk for immunocompromised patients. The taxonomy of these organisms has received little attention though it is increasingly recognised that strains assigned to the taxon exhibit variation in phenotypic properties. Additionally, strains acquired by humans vary in pathogenicity. In the present study, representative strains isolated from clinical and environmental sources were examined for a range of phenotypic properties, including chemical markers. Resulting data show that the strains fell into numerically circumscribed clusters which show some association with clinical relevance.

SE 02 The *Mycobacterium bovis* genome

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It has been estimated that 50 million cattle worldwide are infected with *Mycobacterium bovis* with resulting economic losses of approximately \$3 billion. There is a clear need for the identification of new diagnostic reagents, vaccines, and typing targets if these figures are to be reduced. The *M. bovis* genome project was initiated to accelerate research in all of these areas. Sequencing has revealed that the genome is smaller than that of *M. tuberculosis*, with comparative genomics exposing deletions that impact on a range of metabolic functions. Analysis has shown that the genes encoding the PE and PPE protein families are the most variable loci between the species, suggesting a mechanism for antigenic variation. Genes specific to *M. bovis* have also been identified that may be responsible for the virulence and host range of the bacillus. From an evolutionary perspective it is clear that, contrary to previous thinking, *M. tuberculosis* has not evolved from *M. bovis* and that instead that are derived from a common progenitor. As the study of mycobacterial pathogenicity enters the post-genomic phase the genome of *M. bovis* promises to serve as a cornerstone for studies of comparative virulence, global expression and metabolic profiling.

SE 03 The development of semi-automated fluorescent techniques for improved molecular typing of *Mycobacterium bovis*

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Background: Epidemiological analysis of *Mycobacterium bovis* is complicated by the phenotypic homogeneity of this species. The application of rapid and accurate techniques that exploit genetic

polymorphisms between strains is essential to explore the epidemiology of disease caused by *M. bovis* and the establishment of "global" surveillance systems. Two rapid PCR based fingerprinting techniques, Spoligotyping and fluorescent VNTR (fVNTR), were applied to the analysis of *M. bovis* isolates in Great Britain to determine the suitability of these techniques for use in epidemiological studies of bovine tuberculosis.

Methods: Spoligotyping involved the PCR amplification of the Direct Repeat (DR) region and reverse line blotting of the PCR products against hybridised spacer sequences. fVNTR typing involved the PCR amplification of six loci (A-F) that contain variable numbers of tandem repeats. Fluorescently labelled primers were used for the amplification of each VNTR locus and the PCR products pooled and analysed on an Applied Biosystems 377 DNA Sequencer. This allowed high throughput analysis of samples and semi-automated allele naming. PCR amplification was performed directly on bacterial cells. The data was displayed using Geographical Information software.

Results: Twelve major spoligotypes were observed and of the 3,923 isolates with these spoligotypes, 69.5 % were attributable to one of two spoligotyping patterns, designated type 09 and 17. Spoligotype 09 was found to be widespread throughout England and Wales whereas spoligotype 17 was found in a confined geographical location in south-central England. Molecular and epidemiological data suggests that spoligotype 17 isolates have been derived from spoligotype 09 isolates. VNTR typing gave further differentiation of *M. bovis* isolates from cattle of the same spoligotype and geographical clustering of the VNTR profiles was observed. Greater variation was observed for isolates with spoligotype 09 than for the more geographically restricted spoligotype 17 isolates.

Conclusion: The combined use of spoligotyping and fVNTR for molecular typing of *M. bovis* isolates from Great Britain gives a greater level of discrimination than using each technique individually and is suitable for rapid 'global' analysis of isolates for use in epidemiological studies.

SE 04 A study of a range of antimicrobial agents against rhodococci

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Rhodococci have predominantly been found to cause infection in patients where immunity is reduced by illness or immunosuppressant therapy, however an increase in the number of immunocompetent patients has occurred in recent years. Although a higher frequency of documented cases can be seen, there is still a difficulty for clinical laboratories to identify these pathogens and initiate treatment at the speed required to successfully treat the infection. The present study was designed to aid the compilation of treatment protocols for most types of rhodococcal infections. We performed MIC testing of 160 rhodococci from fifteen validly described species. Testing was performed with 20 antimicrobials in common clinical use. Rhodococci were found to be widely susceptible to glycopeptides, macrolides, rifampicin and many β -lactam antibiotics. Resistance was typically found with methicillin, fosfomicin and aztreonam. Their susceptibility to many other agents was variable and occasionally correlated with species-

type. A comprehensive overview of the susceptibility of this increasingly important genus will be presented.

SE 05 Characterisation of clinically significant rhodococci

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Recent increases in the reported frequencies of rhodococcal infections in humans can be attributed to the widespread use of immunosuppressive drugs and increased clinical and microbiological awareness. Nevertheless, rhodococci are still often missed or misidentified in laboratory specimens, a situation which is not satisfactory because identification of clinically significant rhodococci to the species level is important for establishing the spectrum of diseases caused by members of each species and for predicting antimicrobial susceptibility. The present study was designed to determine the taxonomic status of clinically significant strains isolated from treated and fatal human infections and presumptively identified as rhodococci. The isolates were found to have morphological and chemical properties typical of rhodococci and formed distinct phyletic lines within the zone of evolutionary radiation occupied by the genus *Rhodococcus* thereby adding weight to the view that this genus is underspeciated.

SE 06 Isolation and characterisation of causal agents of mycetoma

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Fifteen strains isolated from clinical cases of actinomycetoma in the Sudan were putatively identified as *Streptomyces somaliensis* on the basis of cultural and morphological properties. All of the isolates contained major amounts of LL-diaminopimelic acid and had phenotypic properties typical of streptomycetes. Complete sequences of the 16S rDNA of the isolates and representative strains labelled *Streptomyces somaliensis* were compared with corresponding sequences of marker streptomycetes. The isolates from the Sudan formed a distinct phyletic line with one of the *S. somaliensis* reference strains; three other *S. somaliensis* markers strains (including the type strain) formed single-membered clades in the 16S rDNA streptomycete tree. The Sudanese isolates can be distinguished from all of the validly described species of *Streptomyces* hence it is proposed that they be recognized as a new species of *Streptomyces*, namely *Streptomyces sudanensis* sp. nov.

SE 07 In vitro versus in silico fluorescent amplified-fragment length polymorphism (FAFLP) analysis of *Mycobacterium tuberculosis*

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The worldwide resurgence of tuberculosis (TB) and the problem of drug-resistant strains have brought *Mycobacterium tuberculosis* back into prominence. Its genome lacks heterogeneity and isolates cannot be resolved by phenotypic strain typing, therefore molecular typing methods are used. We have used the whole-genome fingerprinting technique FAFLP, to identify polymorphism in different strains of *M.tuberculosis*. In the previous study by Goulding *et al* (2000), FAFLP was able to differentiate 65 clinical isolates of *M.tuberculosis*. However, this study only included fragments up to 500 base pairs (bp) in size, whereas we have investigated fragments up to 1000bp. The 1000bp analysis was better than the 500bp, as we have identified more polymorphisms in 500-1000bp fragments in our preliminary study on 10 clinical isolates.

We have investigated *in vitro* FAFLP on H37Rv and CDC1551 strains of *M.tuberculosis* and compared the fragments obtained to that of an *in silico* digest of the respective genomes. The complete genome sequences of *M.tuberculosis* H37Rv and CDC1551 were analysed using TIGR's 'Restriction Digest Tool' (www.tigr.org). Data concerning the size and predicted number of fragments following an *MseI/EcoRI* digest of the genome were imported into a spreadsheet where the fragment size data was adjusted to allow for addition of primer sequences during PCR, before comparing the predicted *in silico* fragments to the *in vitro* results. Overall the *in vitro* and *in silico* data match, although some of the predicted fragments were missing from the *in vitro* results.

SE 08 Design, construction and validation of a whole genome DNA microarray for *Mycobacterium tuberculosis*

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The demand has never been greater for tools to convert the *M. tuberculosis* genome sequence into a greater understanding of the general biology and complex host-pathogen interactions of this pathogen. To address this need our group have undertaken the task of generating a whole genome DNA microarray to facilitate analysis of global gene expression profiles of wild-type and defined mutants of *M. tuberculosis* grown *in vitro* and *in vivo* as well as enabling genotyping studies.

An approach using gene-specific primers has been employed in construction of the *M. tuberculosis* DNA microarray. Using the genome sequence a set of primers were designed to generate PCR products for each of the 3,924 predicted protein coding sequences. The fact that around 10% of the predicted coding sequence belongs to the PE and PPE gene families meant a strategy was required to design the most informative set of PCR products that could differentiate between family member genes with shared regions of homology. To achieve this goal development of the Microarray Design (MAD) software included a procedure that performed BLAST analysis of potential PCR products against the predicted genes. An algorithm was written to select a PCR product for each gene that was unique and only self-detected in the BLAST analysis. If a unique PCR product was

not possible for a particular gene then the algorithm selected a PCR product from the BLAST analysis that demonstrated minimal cross-hybridisation with other non-target genes. This strategy ensured that a particular DNA/RNA species in the labelled sample would only hybridise to a single or minimal number of targets immobilised on the slide, thus maximising the global analysis of the whole *M. tuberculosis* genome. Verification of this strategy has been undertaken by using an *M. tuberculosis* BAC library to investigate the amount of cross-hybridisation observed on the array for genes containing varying degrees of sequence homology.

SE 09 Expression profiling of *M. bovis* BCG and *M. tuberculosis* using a DNA microarray

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Bacterial virulence may be regarded as the correct temporal and spatial regulation of gene expression in response to specific environmental cues. Construction of an *M. tuberculosis* gene-specific whole genome DNA microarray has enabled the global gene expression of this pathogen to be studied in numerous *in vitro* and *in vivo* environments.

Monitoring fluctuations in gene expression during a time course of *M. tuberculosis in vitro* growth, clusters of genes expressed in a similar manner have been identified which may be linked to proposed gene function. Using heat shock as a model system for regulated gene expression, comparative transcriptional profiles of BCG and *M. tuberculosis* have also been examined with the microarray. Comparative genomics has revealed gene deletions in *M. bovis* and *M. bovis* BCG compared to *M. tuberculosis*, with a disproportionate loss of regulatory genes in BCG, the attenuated vaccine strain. The effects of deletions and altered regulatory networks on expression profiles as well as the inter- and intra-experimental variation are quantitatively analysed. Total RNA extraction methods are validated with arrays to highlight the requirement for efficient procedures for obtaining meaningful mRNA populations rather than those reflecting preparation artefacts.

SE 10 An investigation into the expression and regulation of mycocerosic acid synthase (*mas*) in *M. tuberculosis* and *M. bovis* BCG

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Mycocerosic acid synthase (*mas*) is involved in the synthesis of phthiocerol dimycocerosate (PDIM) which plays an important role in the structure of pathogenic mycobacterial cell walls. Recent evidence has shown that this lipid is exported from the bacterium, and is required for growth in the mouse lung. Mycocerosic acid synthase has also been implicated in the survival of *M. smegmatis* in stationary phase growth. Using northern blotting and primer extension *mas* was found to be

upregulated in macrophage derived BCG. *M.tb* and *M.bovis* BCG knockout mutants of *mas* were created by homologous recombination. The knockout mutants contain a deletion of 1247bp within 1.6Kb of the *mas* start codon. Phenotypic characterisation of the *mas* knockout mutants is in progress. The transcription start point of *mas* was identified 178bp upstream of the *mas* ATG and a putative -10 promoter elucidated.

TUESDAY 11 SEPTEMBER 2001

The Promega prize scheme aims to encourage both communication skills and technical excellence in young scientists. The two best presentations in this session will win £200 each.

1405 Molecular assessment of the microbial community in a biopile undergoing bioremediation
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 The *ex situ* remediation of benzene, toluene, ethylbenzene and xylene (BTEX) contaminated sediment from a paint manufacturing site was the focus of this study. Biodegradative activity of the microbial community associated with the *ex situ* remediation of a 5000 m³ biopile was studied over a 130 day period. Changes in biodegradative activity were linked to temporal changes in the microbial community. Bioremediation potential was determined through radiolabelled pollutant mineralisation and selective microbiological counts. Rates of ¹⁴C 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 numbers of toluene degrading bacteria were seen to remain at a constant level. DNA was extracted from soil samples taken at intervals and 16 S rRNA encoding genes amplified by PCR. 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 achieved a more stable level of diversity concurrent with low mineralisation rates and the detection of low levels of BTEX contaminants. Biopile samples were also analysed by PCR-DGGE using primers for the functional *todC1* gene. This gene encodes the catalytic subunit of toluene dioxygenase that is involved in aromatic hydrocarbon degradation. The initial diversity of the toluene degrading community was seen to be low, relative to overall microbial diversity. This diversity was shown to further decrease during the bioremediation process resulting in only a few dominant members remaining notably different from those seen at day 0. The decrease in diversity could be due to selection of the most efficient toluene degraders during the remediation process.

1420 Selective isolation of *Amycolatopsis* strains from environmental samples

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There are currently thirteen validly described species in the genus *Amycolatopsis* 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 rare and novel members of the genus from natural habitats. The aim of the present study was to develop an effective selective procedure for the isolation of industrial significant novel strains of *Amycolatopsis* and to study their geographical distribution. Large numbers of *Amycolatopsis*-like colonies were isolated from diverse environmental samples following the incubation of soil suspensions on media supplemented with antimicrobial agents. 16S rDNA sequence studies of representative strains showed that they form new centres of taxonomic variation within the genus *Amycolatopsis*. The integrity of these taxa was underpinned by additional genotypic and phenotypic data and representative strains were screened for novel metabolites.

1435 Development of an immuno-PCR assay for the detection of mumps-specific IgG

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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 (I-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 I-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.

1450 DNA array analysis of murine gammaherpesvirus 68 transcriptional profiles

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Murine gammaherpesvirus-68 (MHV-68) is 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 predicted open reading frames as well as inter-gene sections. Using this technique, progression through the virus' life cycle has been observed for the first time from a transcriptional perspective. Such experiments have allowed characterisation of each gene by its expression profile. Transcripts were also categorised into α -, β - and γ -kinetic classes by blocking *de novo* protein synthesis and DNA replication *in vitro* with cycloheximide and 2'-deoxy-5-ethyl- β -4'-thiouridine respectively.

The MHV-68 array allows changes in gene expression to be analysed rapidly at the genome level. By characterising mutant viruses we can examine the knock-on effects of knocking out single genes. By moving *in vivo*, we can examine the programme of transcriptional events in disease, and in particular during latency and reactivation. These studies provide insight into the function of the genes in question, as well as their position in the context of global gene expression.

1505 Mining the *Burkholderia pseudomallei* genome and proteome for resistance factors

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Burkholderia pseudomallei is the causative agent of melioidosis in both humans and animals. Melioidosis is endemic in both Southeast Asia and northern Australia and carries a high rate of mortality, 95% if left untreated. Treatment involves prolonged periods of antimicrobial therapy and relapse is common. Better screening and an increase in awareness has led to a rise in the number of reported cases each year in endemic areas such as Thailand and the disease has also been shown to be emerging in other countries such as India and China.

We have initiated studies to map the extracellular proteome using 2D gel electrophoresis / mass spectrometry analysis. The recent availability of the genome sequence is an essential component of these studies for the assignment of spots to specific open reading frames and genes. β -lactamase enzymes were chosen to act as potential marker proteins whose expression levels would be expected to change during different growth conditions such as Augmentin stress. Mining for potential β -lactamase genes was carried out using the sequences of closely related organisms and the genome sequence BLAST search facility. Four β -lactamase enzymes have been identified: a Class A, Class B1, Class C and Class D enzyme. We have now initiated studies involving the analysis, isolation and characterisation of these enzymes.

1550 Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription and cleavage by metalloprotease

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The fibrinogen binding protein ClfB of *S. aureus* was shown to be expressed on the cell surface predominantly in the exponential phase of growth. It decreased throughout the growth cycle and was barely detectable on stationary phase cells. Expression studies using a *clfB::lacZ* fusion indicated that transcription stopped before the end of the exponential phase of growth. Mutations in the global regulators *agr* and *sarA* had no effect on *clfB* transcription and the possibility of a novel regulator is currently being examined. Two forms of the protein were detected on the cell surface, the smaller of which was generated by the loss of an N-terminal domain by proteolysis. The proportion of the smaller form increased as the cultures grew. The metalloprotease, aureolysin (*aur*), was found to be responsible for cleavage of ClfB. Cleavage was inhibited by EDTA and o-phenanthroline and did not occur in an aureolysin-deficient mutant. Purified aureolysin cleaved cell-surface located ClfB as well as the recombinant A-domain of the protein removing 180-182 residues from the N-terminus at a motif SLAVA. The truncated form of ClfB did not bind to fibrinogen. Transcription studies using an *aur::lacZ* fusion indicated that expression occurred in early exponential phase, consistent with truncation of ClfB at this time, and confirmed that the *aur* gene is negatively regulated by the SarA protein.

1605 Bacterial cytoskeleton: cell shape determination in *Bacillus subtilis*

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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.

1620 Identification of novel tetracycline resistance determinants harboured by anaerobic gut commensal bacteria

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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 designated TnB1230 in *B. fibrisolvens* 1.230. Sequence analysis of TnB1230 has identified several open reading frames with homology to transfer functions encoded by conjugative elements from important human pathogens. Genes > 99% identical to *tet(W)* were also isolated from a low G+C Gram positive human colonic anaerobe K10, and from *Bifidobacterium longum* F8.

Tetracycline resistance was transmissible from the human isolate K10 to the rumen anaerobe *B. fibrisolvens* 2221^R *in vitro*, however *tet(W)* was not detected in the transconjugants. Instead a second novel Tc^R gene designated *tet(32)* was shown to be transmissible from K10. This gene has also been characterised and fully sequenced.

The environmental distribution of these new genes and in particular their possible contribution to previously unidentified tetracycline resistance amongst pathogenic bacteria requires further investigation.

1635 Upstream regulation of *actII-ORF4*, a key activator of antibiotic production in *Streptomyces coelicolor*

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Streptomycetes produce antibiotics in response to environmental triggers. Expression of genes for antibiotic biosynthesis is usually activated by a pathway specific protein. Actinorhodin biosynthesis in *S. coelicolor* is activated by ActII-ORF4, and streptomycin biosynthesis in *S. griseus* by StrR. These pathway specific regulators belong to different regulatory protein families. We aim to elucidate the regulatory protein cascade that links environmental changes to the production of ActII-ORF4.

S. coelicolor protein extracts contain a single component that binds specifically to two separate regions flanking the transcriptional start point of *actII-ORF4*. Interestingly, the same component also binds upstream of *S. griseus strR*. The protein responsible is being purified using this specific binding as an assay; partial purification should permit identification of candidate *S. coelicolor* gene products. Binding site sequences are being determined by copper-phenanthroline footprinting. Existence of a binding activity common to *actII-ORF4* and *strR* suggests that the mechanisms regulating the production of antibiotic pathway-specific activators in disparate streptomycetes may be evolutionarily conserved, even though the final activators are not.

1650 Biotransformations of morphine alkaloids by cytochrome P450

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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 synthesis 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 further aim to engineer these cytochromes P450 to increase their activity and specificity towards the opioids, then introduce the activity into various organisms, for specific biotransformations as well as pathway engineering towards the industrial production of semi-synthetic opiate drugs.

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Cells & Cell Surfaces Group

Thursday 13 September 2001

1400 Cell surface requirements for *Myxococcus* multicellular development

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Cell-cell interactions and signal transduction are critical to all aspects of the *Myxococcus xanthus* life cycle, particularly its multicellular fruiting body development that is initiated by starvation at high-cell density. As a result, the *M. xanthus* cell surface is a focal point for studies of the regulation of the developmental program. The absence of the lipopolysaccharide (LPS) O antigen has been shown by suppressor analysis to bypass the high density (A-signal) requirement for a class of early developmental reporter genes represented by the *spi* gene (previously designated 4521). Dialysis experiments have shown that this stimulation is an intrinsic property of these mutants, rather than being due to an increase in permeability to A-signal. Epistasis experiments suggest that the absence of LPS O antigen stimulates the SasS sensor kinase that controls *spi* expression. Specific suppressors of this LPS O-antigen-stimulated *spi* activity, which return *spi* expression to a more wild-type level, map to a xylose-biosynthesis locus. These mutations alter the LPS core and the other surface-associated polysaccharide material termed fibrils. We propose that the block in LPS O-antigen biosynthesis results in the accumulation of intermediates in the periplasmic space that is sensed by the SasS sensor kinase leading to *spi* overexpression. Furthermore, the suppression by the xylose biosynthesis mutations is thought to relieve the stimulation of SasS by blocking the accumulation of the intermediates.

Previous work in our laboratory showed that the *M. xanthus* LPS O-antigen is required for gliding motility. Specifically it is necessary for social (S) motility, as judged by the smooth colony-edge phenotype of double mutants in adventurous (A) motility and LPS O-antigen biosynthesis. Further analysis of the *M. xanthus* LPS O-antigen mutants indicates that they are defective in both the A and S motility systems, showing drastically reduced gliding speeds at high density and poor colony spreading on an agar/air interface. Interestingly, the LPS O-antigen mutants are defective in slime trail deposition, but the presence of wild-type slime trails does not rescue the phenotype. These results suggest that the role of the LPS O antigen in gliding motility is not confined to modification of the substratum. We propose that the release of LPS O antigen plays an active role in surface translocation.

POSTERS:**MI 29 *Pasteurella multocida* toxin (PMT) induces tyrosine phosphorylation of the heterotrimeric G protein G_{q11}**

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Bacterial toxins are important agents of disease but also form useful tools with which to probe eukaryotic cell biology. The *Pasteurella multocida* toxin (PMT) is a highly potent mitogen for a variety of cell types, and in particular murine Swiss 3T3

fibroblasts. The mitogenic effects of PMT are thought to be mediated through heterotrimeric G proteins of the G_q subfamily. In this current study we demonstrate that PMT induces tyrosine phosphorylation of the alpha subunit of the heterotrimeric G protein G_{q11} using Western Blot analysis. Interestingly, a previously described non-mitogenic mutant of PMT also induces tyrosine phosphorylation with kinetics matching those of the wild-type toxin. PMT mediated sustained phosphorylation of G_{q11} in contrast to the highly transient phosphorylation event induced by the G-protein coupled receptor agonist (GPCR) bombesin. Whilst both wild-type and mutant toxins induce tyrosine phosphorylation of G_{q11}, only the wild-type toxin stimulated the production of inositol phosphates. To further investigate this effect G-protein activation assays were performed. Wild-type toxin and bombesin, but not mutant toxin, stimulated the binding of the non-hydrolysable GTP analogue guanosine 5'-O-(thio) triphosphate (GTPγS) to plasma membrane fractions from Swiss 3T3 fibroblasts. The results presented using wild-type and mutant forms of PMT raise doubts over the previously held hypothesis that tyrosine phosphorylation of the alpha subunit correlates with G-protein activation.

MI 30 Cytotoxic necrotizing factor induces cyclooxygenase-2 expressionWARREN THOMAS¹, ZOE ASCOTT¹, LEE SLICE², ENRIQUE ROZENGURT² & ALISTAIR J. LAX¹¹Oral Microbiology, King's College London, Floor 28 Guy's Tower, Guy's Hospital, London SE1 9RT, ²Dept of Medicine, School of Medicine and Molecular Biology Institute, UCLA, Los Angeles, California 90095

Cytotoxic necrotizing factor 1 (CNF) is a toxin produced by some isolates of *Escherichia coli* that can cause extraintestinal infections. CNF can initiate cell signalling pathways that are mediated by the Rho family of small GTPases through a covalent modification that results in constitutive activation. In addition to regulating the assembly of actin stress fibres and focal adhesion complexes, RhoA can also regulate gene expression at the level of transcription. Using a luciferase based reporter system, we have demonstrated that the transcription of cyclooxygenase-2 (COX-2) is strongly upregulated in NIH 3T3 fibroblasts treated with CNF and that this effect is dependent upon the activation of RhoA by the toxin. The transcription signal is not mediated by Rho associated kinase (p160/ROCK) and so must rely upon another effector which is activated by RhoA. In addition we have shown that subsequent protein tyrosine phosphorylation events modulate the induction. CNF therefore induces COX-2 expression via a RhoA dependent signalling pathway that diverges from that which regulates cytoskeletal rearrangements in response to RhoA activation.

MI 31 Identification of the functional domains of the *Pasteurella multocida* toxin (PMT)

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Pasteurella multocida toxin (PMT) is a potent mitogen. It is a large polypeptide (146 kDa) which acts intracellularly. It activates G_q, though its molecular mode of action is unknown. We predict that PMT will comprise domains for cell binding, membrane translocation and catalytic activity. PMT is

homologous with the cytotoxic necrotizing factor (CNF) of *E. coli* towards the N-termini of both toxins. The N-terminus of CNF is required for cell-binding and internalization, and the C-terminus for activity. We previously identified PMT amino acid 1165 (which is close to the C-terminus) as necessary for activity, and postulated that the C-terminal region contained the catalytic domain. In this study, a series of N- and C-terminal fragments were expressed as GST- or hexahistidine- fusion proteins, and the effects of the purified peptides were assessed in assays for catalytic activity and cell-binding. Cell-binding was investigated by setting up a competition assay. An N-terminal fragment reproducibly blocked PMT activity when added at a 10^4 molar excess, suggesting that it contains the binding domain. The C-terminal half of PMT but not N-terminal fragments reproducibly induced DNA synthesis when microinjected into the cytoplasm of quiescent Swiss 3T3 cells, and led to a cell morphology similar to toxin-treated cells. Furthermore, microinjection of antisera prepared against the C-terminus inhibited the activity of PMT added extracellularly significantly more than pre-immune sera or anti-N-terminal Ab. This is the first direct evidence that the C-terminus of PMT has catalytic activity.

PBMG 16 Flux balance analysis of *Saccharopolyspora erythraea*

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Flux balance analysis has been applied to many aspects of primary metabolism in simple microorganisms such as *E. coli*. However certain aspects of secondary metabolism make it far less accessible to flux balance analysis. The work here represents an attempt to address flux balance analysis to secondary metabolism. New approaches have been identified and applied to the secondary metabolism of *Saccharopolyspora erythraea*. To achieve this a metabolic network has been constructed that takes into consideration the possibility of accumulation and breakdown of storage compounds and other cellular components. The changing composition of the biomass has been measured in batch culture under carbon and nitrogen limitation. This information has been used to inform the flux balance model on the production and utilisation of biomass components linked to secondary metabolism. The effect of changes in biomass composition on flux balance analysis is discussed.

PBMG 17 Changing the carbon flux through the fermentation pathways of *Bacillus stearothermophilus* by metabolic engineering

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Bacillus stearothermophilus is a thermophilic facultative anaerobe which has been investigated as a candidate for ethanol production from plant biomass. During fermentation *B. stearothermophilus* produces significant amounts of lactate via an NAD⁺-linked lactate dehydrogenase (LDH) as well as minor amounts of formate, acetate and ethanol.

A strain of *B. stearothermophilus* has been metabolically engineered to increase the yield of ethanol produced by knocking out LDH activity. This directs carbon flux away from lactate production which results in the majority of carbon flux during fermentation being directed towards ethanol. In addition,

heterologous pyruvate decarboxylase (PDC) from *Zymomonas mobilis* has been introduced to the LDH⁻ mutants to increase ethanol yields close to the theoretical maximum.

These changes in metabolic flux have been monitored in the wild type, LDH⁻ mutants and the LDH⁻ PDC⁺ mutants of *B. stearothermophilus* when grown in continuous culture under metabolic stress due to excess carbon source. The changes observed in metabolic flux in the fermentation pathways of the mutants and wild type *B. stearothermophilus* will be discussed.

