

# Proceedings of the 8th European Oral Microbiology Workshop

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The meeting place for the Eighth European Oral Microbiology Workshop was the Sani Resort on the Halkidiki peninsula of Kassandra in Northeastern Greece. The Workshop was organized by Professor Sotos Kalfas and colleagues from the Dental School at Aristotle University of Thessaloniki. Sixty-four delegates from 12 European countries, the USA, and Japan attended the meeting. The workshop was organized into ten oral sessions over two days. A new feature of this European Oral Microbiology Workshop was a poster session, for which there were 21 presentations. These proceedings summarize presentations and debate within the ten oral sessions covering many aspects of oral microbiology.

## Biofilms in Dentistry

The first session, chaired by Phil Marsh (Leeds Dental Institute, UK), dealt with a range of contemporary and wide-ranging issues on dental plaque as an example of both a biofilm and a microbial community. Howard Jenkinson (Bristol Dental School, UK) posed the question, "Do signals matter?" Evidence was presented in relation to the interference of signals that may disrupt biofilm formation in terms of how bacteria respond to their environment and interact with each other. Reference was made to the *com* and *cia* regulatory circuits used by *Streptococcus* spp. (Wang and Kuramitsu, 2005). Discussion related to why signals may not be important in the environment, although shown to be active in the laboratory, and what benefit there is in the recognition of a signal from a different bacterium. The debate considered the relative importance of cell-cell communication *vs.* more efficient metabolism in a biofilm community. William Wade (King's College, London, UK) suggested that there is a rationale for the whole biofilm community to be under control through the use of signaling mechanisms. Bernie Guggenheim (University of Zürich, Switzerland) suggested that metabolic interactions might be of overriding importance. The next part of the session concentrated on the penetration of molecules into, and effect on microbial diversity within, biofilms. David Beighton (King's College, London, UK) described how acid stress could increase habitat heterogeneity, with subsequent increase in species and genotype diversity. He indicated that, in active caries sites, there is an unexpected increase in population diversity within dental plaque. Discussion focused on the extent of diversity changes within an acidic environment, and how bacteria may migrate in response to an acid gradient. Two presentations then followed on the penetration of molecules within biofilms. Bernie Guggenheim and Phil Watson (Masterfoods, UK) presented data from the Zürich *in vitro* and Leeds *in vivo* models, respectively. Bernie argued that his six-species biofilm model demonstrated that molecules move through pores (2.6-4.6 nm in diameter) rather than channels, and that he, in

contrast to others, considers biofilms to be *hydrogels*. He also pointed out that ineffective staining of the plaque extracellular matrix may demonstrate the presence of channels. Discussion centered on the relevance of the model with respect to nutrient supply and antimicrobial resistance. Phil Watson then presented data on the penetration of fluoride and triclosan through a biofilm, using the 'Leeds *in situ* device' (nylon ring held on the tooth surface) (Watson *et al.*, 2005). It has been shown that duration of exposure, delivery vehicle, and saliva washing all influence uptake. Elin Giertsen (University of Bergen, Norway) presented work on the effect of xylitol on the survival of mutans streptococci in biofilms, using xylitol-resistant and -sensitive strains. While *in vivo* studies offer support that xylitol can reduce/eliminate these bacteria, *in vitro* studies do not. Sigrun Eick (Jena University Hospital, Germany) then continued with a presentation on the *in vitro* efficacy of chlorhexidine against periodontal bacteria within a multi-species biofilm flow system with synthetic saliva. Phil Marsh pointed out that species/strains of bacteria used in such systems are critical in terms of interaction with each other. Ellen Frandsen (Aarhus University, Denmark) then opened the discussion on biofilms associated with dental unit water systems (DUWS), and the suggestion that Europe should adopt formal guidelines on the microbial quality of such water, analogous to the American Dental Association guideline of 200 colony-forming units/mL. Gunnar Dahlén (Göteborg University, Sweden) remarked on the low incidence of known problems relating to DUWS quality with reference to reported *Legionella* and *Pseudomonas* infection. Further discussion on the approach to DUWS contamination related to hygiene and education issues.

## Bacterial Adhesion and Host Susceptibility

Nicklas Stromberg (Umeå University, Sweden) introduced the second session, relating to the prerequisite ability of oral bacteria to adhere to be able to colonize surfaces within the oral cavity, and recent advances in understanding how the host influences microbial colonization. In some circumstances, genotypic and phenotypic variations within the host may have implications for changes from health to disease. The conditioning film or pellicle greatly influences the initial colonization of surfaces within the oral cavity, and work from Stromberg's own laboratory has indicated that host susceptibility to dental caries may be due to expression of a 'Db' variant of the salivary proline-rich protein (Prp), known to be an important component of the enamel pellicle. The Db variant Prp is preferentially bound by caries-causing *Streptococcus mutans*, rather than by commensal streptococci such as *S. gordonii*, which bind the more common Prp1 and Prp2 as initial colonizers (Stennud *et al.*, 2001). Individuals expressing the Db variant are therefore more likely to harbor *S. mutans*, and to have a higher incidence of caries. Another intriguing aspect of Prps is a role in innate immunity specified through an internal pentapeptide, released following

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Oral microbiology, biofilms, adhesion, taxonomy, uncultivable, virulence, host defense peptides, host response.

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hydrolysis by streptococcal endopeptidases. This pentapeptide has several properties which may influence early-colonizing bacteria, e.g., stimulation of growth rate of commensal streptococci, inhibition of desorption, and interference in bacterial metabolism, as indicated by the inhibition of pH drop following exposure to sucrose (Li *et al.*, 2000). This may have an impact on enamel dissolution at the local level. David Beighton asked if the concentration of this pentapeptide in dental plaque is known, and, if so, whether the levels of the pentapeptide used in laboratory experiments are physiologically relevant. Pentapeptide concentrations in plaque are unknown, making judgment of the levels for use in *in vitro* studies difficult. The specificity of the pentapeptide sequence is paramount. Peptide molecules with amino-acid substitutions are reduced or altered in activity. Anette Carlén (Göteborg University, Sweden) provided information on *in vivo* enamel pellicle composition 60 minutes after tooth-surface polishing, comparing gingival and incisal samples from both healthy individuals and those with inflamed gingiva. More fibrinogen, fibronectin, albumin, and IgG have been detected in the pellicle of healthy individuals, but IgA levels were similar in both groups. Bacteria were also sampled, and more periodontopathic organisms were detected in those individuals with inflamed gingiva. It appeared that plasma proteins could be considered important components of the enamel pellicle, in addition to the well-established presence of salivary proteins. Howard Jenkinson reminded the audience that glucosyl-transferases were also important components of the pellicle, and Bernie Guggenheim emphasized that particles of dietary foodstuffs also became incorporated within the matrix. Howard Jenkinson then presented information relating to surface structures of oral streptococci and interactions with the high-molecular-weight glycoprotein, gp340. The Antigen I/II family is composed of serine-rich surface proteins that are widely distributed in the oral streptococci, with around 60-80% identity between those expressed by *S. gordonii* and those expressed by *S. mutans*. *S. mutans* relies on Antigen I/II for binding to gp340, and mutants are abrogated in this interaction (Jakubovics *et al.*, 2005).

Another family of surface proteins is also widespread, but is not found in mutans-group streptococci. These Sxr proteins are highly glycosylated and are important in binding gp340, and mutant strains of *S. gordonii*—which have been deleted for the Sxr protein, Hsa—will not bind gp340, and are compromised in their ability to form a biofilm. *S. gordonii* recognizes and binds sialic acid in gp340 carbohydrate side-chains, and has at least two surface adhesins (Ag I/II and Hsa) for binding this molecule, whereas *S. mutans* binding is not affected by prior treatment of gp340 with sialidase, and Ag I/II is the principle adhesin. Thus, both organisms recognize and bind gp340, but with different adhesin profiles and specific targeting within gp340. Aggregation of *S. gordonii* is facilitated by gp340. The wild-type and Hsa-deleted mutant both aggregate rapidly when mixed with gp340, whereas an Ag I/II mutant has reduced aggregation (Loimaranta *et al.*, 2005). Bacteria therefore interact differently with fluid-phase gp340 compared with surface-adsorbed molecules, probably due to conformational changes of the ligand. Mogens Kilian (Aarhus University, Denmark) enquired whether it was known if sialidase activities expressed by oral bacteria affected interactions with gp340. This is as yet unknown, but within the pneumococcal genome there are three genes encoding putative sialidases, and pneumococci release a neuraminidase from the cell surface, suggesting an interplay between free and cell-bound sialic acid. gp340 is found in parotid saliva in the oral cavity, but is reported from other sites within the human body, e.g., the lung. Georg Conrads (Aachen University Hospital, Germany) reported that gp340 was known to neutralize HIV. Ian Douglas (University of Sheffield, UK) continued the theme, describing studies investigating the role of SrpA, a Sxr-family

protein expressed by *S. sanguis*, an oral organism frequently associated with infective endocarditis, in binding to the platelet glycoprotein, GPIb (Plummer *et al.*, 2005). A video clip was shown of platelets flowing over, and adhering to, bacterial cells. An SrpA inactivated mutant is reduced in GPIb binding. SrpA function in binding GPIb may be an accident of evolution, whereby the molecule may normally have evolved to bind sialylated glycoproteins, such as mucin glycoprotein 2 (MG2) or gp340, but once access is gained to the bloodstream, it has the capacity to interact with GPIb, and therefore plays a role in bacterial colonization of platelet-fibrin vegetations within the endocardium. Howard Jenkinson asked whether the interaction between platelets and cells indicated a 'catch hook' attachment, which Ian confirmed. The assay had to be undertaken with bacteria on the surface rather than platelets, since these became activated on contact with a surface. Niklas Stromberg pointed out that GPIb was essential for wound healing. Jacob ten Cate (ACTA, Amsterdam, Netherlands) enquired which von Willebrand factor (VWF) bound GPIb, and Ian replied that most did.

### Oral Bacterial Infections and Systemic Diseases

The third session on oral bacterial infections and systemic diseases, chaired by Sirkka Asikainen (Umeå University, Sweden), was organized with reference to the different possible mechanisms by which oral bacteria may have an impact on systemic disease. In terms of the prevalence of potentially oral-derived bacteria in non-oral infections, oral streptococci (30-40%) and the HACEK group (*Hemophilus* spp., *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella* spp.) (5-10%) are considered to be significant. Wil van der Reijden (ACTA, Amsterdam, Netherlands) made reference to studies used to type *A. actinomycetemcomitans* and *Prevotella intermedia* from oral and non-oral sites within the same individual. Eija Könönen (National Public Health Institute, Finland) presented findings from a study to observe the relationship between the oral (saliva) and nasopharyngeal (aspirate) flora during the first 2 years of life. Obligate anaerobes were found in 92% and 2% of oral and nasopharyngeal samples, respectively. Oral streptococci were found in 100% and 60% of samples, respectively. She also described studies using AP-PCR to determine clonal similarity between *Fusobacterium nucleatum* isolates from the oral cavity and those from the nose (Haraldsson *et al.*, 2004). Mogens Kilian remarked on the possibility of salivary contamination when taking nasopharyngeal samples. Sirkka then introduced her presentation with reference to the possible systemic release of components of the oral microflora with biological properties. She described experiments to examine the release of peptidoglycan-associated lipoprotein and lipopolysaccharide from *A. actinomycetemcomitans* into serum. Rob Allaker (Queen Mary, UK) then reviewed some of the studies that suggested an association of *Fusobacterium nucleatum* with pregnancy complications (Han *et al.*, 2004). Comments related to the possibility that given subspecies/clonal types may be more likely to spread systemically. Sirkka then introduced the concept that oral bacteria/oral infection may contribute to atherogenesis and promote development of cardiovascular disease. Susanna Paju (University of Helsinki, Finland) described a study that demonstrated a correlation among matrix metalloproteinase-9, periodontal disease status, and serum antibodies to periodontal bacteria in patients with acute coronary syndrome. The possible relationship between periodontitis and the structure/function of LDL and HDL cholesterol molecules was then discussed. The isolation of these molecules from individual periodontitis patients has allowed macrophage stimulation by LDL cholesterol and

changes in the phospholipid composition of HDL to be determined. It was concluded that, to avoid such potential contribution to heart disease, periodontitis should be treated as early as possible, or prevented.

## Recent Developments in Taxonomy

Anne Tanner (Forsyth Institute, Boston, MA, USA) chaired the fourth session, which provided an update on recent major changes in taxonomy of oral bacteria. William Wade presented a summary of current taxonomic status of oral microorganisms. 16S rRNA phylogenetic comparisons remain a most useful tool for such studies, although not appropriate or particularly useful in some taxa. The audience was reminded that there are three domains of life—the *Eukarya*, *Archaea* (with one oral genus of *Methanobrevibacter*), and *Bacteria*. The session focused mainly on *Bacteria*. Cultivable oral bacteria traditionally defined as 'Gram-negative' are classified into the phyla *Bacteroidetes* (within which are the genera *Bacteroides*, *Porphyromonas*, *Tannerella*, *Prevotella*, and *Capnocytophaga*), *Spirochetes* (*Treponema*), *Synergistes* (*Synergistes*, *Desulfothiovibrio*), *Proteobacteria* (*Neisseria*, *Hemophilus*, *Actinobacillus*, *Campylobacter*, *Eikenella*, and *Desulfobulbus*), and *Chlamidiae* (*Chlamidophila*). Oral treponemes are well-known as being particularly difficult to culture *in vitro*. The *Synergistes* provide another example of high frequency of molecular detection, which is not reflected by cultural data, on analyses of oral samples. Molecular detection techniques indicate bacteria within this phylum to be very common in dentoalveolar abscesses, and Georg Conrads indicated that 80% of intra-abdominal infections contain these bacteria. Those oral bacteria habitually described as 'Gram-positive' consist of five cultivable phyla. These include the *Actinobacteria*, which may previously have been known as the high mol% GC Gram-positives, and within which are the following genera—*Olsenella*, *Atopobium*, *Slackia*, *Eggerthella*, *Bifidobacterium*, *Scardovia*, *Parascardovia*, *Cryptobacterium* (all within the family *Coriobacteriaceae*), *Propionibacterium*, *Corynebacterium*, *Rothia*, *Actinomyces*, and *Actinobaculum*. The low mol% GC Gram-positives are now classified within the phylum *Firmicutes*, and include the genera *Streptococcus*, *Abiotrophia*, *Gemella*, *Catonella*, *Granulicatella*, *Lactobacillus*, *Dialister*, *Peptostreptococcus*, *Filifactor*, *Eubacterium*, *Mogibacterium*, *Pseudoramibacter*, *Propionibacterium*, *Bacillus*, *Shuttleworthia*, *Bulleidia*, *Selenomonas*, and *Veillonella*. Other 'Gram-positive' phyla with oral representatives include *Fusobacteria* (including the genera *Fusobacterium* and *Leptotrichia*), *Deinococcus-Thermus* (genus *Deinococcus*), and *Acidobacteria* (genus *Acidobacterium*). Of these, it should be noted that *Selenomonas*, *Veillonella*, *Dialister*, *Fusobacterium*, and *Leptotrichia* stain Gram-negative. Mogens Kilian provided a summary of the current status of oral species within the streptococci, pointing out the difficulties of using 16S rRNA sequence data for phylogenetic classification. A good example of this was found in *mitis*-group streptococci, in which different rRNA operons within the same genome may contain either typical *oralis*-like or *mitis*-like sequences, presumably reflecting acquisition of 16S rRNA genes by transformation and recombination. Sequencing housekeeping genes is another approach that has been used to overcome such problems. Commercially available kits are commonly used for identification of streptococcal isolates. However, a recent survey has reported only 12.5%, 35%, 63%, and 50% correct identification for *S. mitis*, *S. oralis*, *S. sanguis*, and *S. mutans*, respectively, using one of these kits. Nonetheless, identification of *S. anginosus* isolates was excellent using this same system. Mogens also repeated a plea for the traditional nomenclature for streptococcal species to be retained, but Phil Marsh suggested that different groups using different streptococcal names would only lead to confusion. Adoption of new or corrected names may be confusing for a few years,

but a universally adopted nomenclature will ultimately lead to clarity. (Postscript: Since this meeting, Mogens has contacted all workshop participants to confirm, after further consultation, that the new, and not the traditional, nomenclature, *must* be used—the old names, with incorrect Latin grammar, are considered 'illegitimate'.) Julia Downes (King's College, London, UK) presented on phylogenetics of the Peptostreptococci. Analysis of 16S rRNA sequences had indicated that non-oral isolates of *P. anaerobius* had a deletion in the V1 region in comparison with oral isolates. There followed general discussion on the relative merits of phylogenetic methods, with an agreement that no single method was ideal, and that polyphasic approaches should be considered. Changing bacterial names could be confusing for clinical colleagues, and it was felt that a user-friendly alert system, for notification of both classification changes and emergence of disease-associated bacteria, would be useful.

## Unculturable Oral Bacteria

William Wade chaired this fifth session, and introduced the topic with a brief overview of molecular methodologies that, allied to microscopic observations and cultural data, were now providing understanding of unculturable oral bacteria. Around 50% of the oral flora is thought to be unculturable thus far. Thirteen of 37 bacterial phyla have no culturable isolates. Taxa within the TM7 phylum are common but cannot be cultured, although they are known to have a Gram-positive cell wall. Anne Tanner presented data indicating that 'not-yet-cultured' bacteria were readily detectable by 16S rDNA checkerboard from oral samples obtained from healthy individuals, and from those with early periodontitis, either on the tongue or in subgingival plaque. Ian Douglas enquired whether culturable bacteria known to be within a sample were ever undetected by molecular methods such as 16S rDNA PCR. William Wade confirmed that 16S rRNA genes with long GC sequences may be difficult to amplify by PCR, and that the organisms from which these genes were derived may therefore not be detected. Rob Whiley (Queen Mary, UK) asked whether attempts were being made to improve culture of 'unculturable' bacteria. Mixed cultures allowed for the growth of some of these bacteria, with the concerted activities (metabolic or otherwise) of the diverse flora contributing to this success. In some cases, two bacterial strains could be separated by a porous barrier, *e.g.*, dialysis tubing, with low-molecular-weight molecules traversing this barrier to assist the growth of 'unculturable' bacteria. William speculated that some bacteria that are unculturable require some form of resuscitation, perhaps requiring a signal for growth, and relating to a cytokine network for switching growth on and off. A heat-resistant, < 3 kDa component of a sonicate of *Fusobacterium nucleatum* cells was one such stimulator of some unculturable bacteria. This component has not yet been identified. A paradigm for similar resuscitation is known in medical microbiology. An extracellular 'resuscitation promoter factor' from *Micrococcus luteus* stimulates growth of *Mycobacterium tuberculosis* at concentrations as low as 10<sup>-15</sup> mol (Freeman *et al.*, 2002). This factor may be a useful adjunct to conventional therapeutics for control of this organism, since the non-stimulated, dormant form is likely to be more recalcitrant to antibiotic control. Melanie Wilson (Cardiff Dental School, Wales, UK) reported on an investigation into microbial content of oral squamous cell carcinomas (SCC). Known risk factors for oral cancers do not account for all cases, and there may be an as-yet-unrecognized microbial contribution to the initiation and progression of SCC. There is already evidence for the role of bacteria in other cancers, *e.g.*, *Helicobacter pylori* and gastric cancers. Samples from controls and from superficial and deep sites in oral SCC had been studied by both microbial culture and 16S rRNA analysis. Six

species of bacteria/sample were detected by culture, and 10 phylotypes by molecular analysis. Novel species had been identified, and *Micrococcus*, *Fusobacteria*, *Prevotella*, and *Veillonella* had been detected within tumor tissues. Rob Allaker pointed out that Micrococci were generally considered to be skin bacteria, and Melanie agreed, but countered that these were found in lesions but not control tissues. The intriguing possibility of a bacterial role in SCC requires further investigation. Georg Conrads described an investigation of incidence and diversity of sulphate-reducing bacteria (targeting *dsrAB*) and methanogens (targeting *mcrA* and 16S rDNA) in patient cohorts exhibiting periodontal health, mild periodontitis, and aggressive periodontitis. Very little microbial diversity was found, but the incidence of both bacterial groups was high in patients with aggressive periodontitis, but not in healthy patients or those with mild forms of periodontal disease.

### Microbial Invasion in Periodontal Lesions

The sixth session, on microbial invasion in periodontal lesions, was chaired by Gunnar Dahlén. He introduced the topic by reviewing the mechanisms—*i.e.*, trauma, growth, motility, and engulfment—by which micro-organisms gain access to periodontal tissues. Sigrun Eick described how *Porphyromonas gingivalis* may modulate the immune response from within epithelial KB cells, through survival and up-regulation of IL8 production. The effect of *P. gingivalis* on KB cell numbers, the use of both invasive and non-invasive strains, and the duration of exposure were points raised by the audience. Mike Curtis (Queen Mary, UK) raised the issue as to why *P. gingivalis* would encourage the up-regulation of IL8, which, in turn, would recruit neutrophils? Ian Douglas then presented results of a study to investigate the role of gingipain in *P. gingivalis* invasion. Using an antibiotic protection assay, he demonstrated that strains varied in their ability to invade epithelial cells. The gene knockout strain E8, which has no arginine-x-specific activity, was shown to be more invasive than wild-type strains. E8 was also shown to bind fibronectin at higher levels than other strains. The possibility of changes within the adhesin domain of gingipain was proposed to explain the differences observed. Nezar Al-Hebshi (University of Bergen, Norway) suggested the use of a stratified model system to complement the observations from the use of monolayer cell cultures. With reference to future studies of invasion by oral bacteria, it was pointed out that microbial consortia would need to be included, since co-operative mechanisms may well operate (Rudney *et al.*, 2005).

### Is Periodontitis a Virus Infection?

In session 7, Minas Arsenakis (Aristotle University, Thessaloniki, Greece) addressed the question, 'Is periodontitis a virus infection?' He introduced the topic by reviewing recent studies that have shown several viruses, in particular human herpes viruses, to be associated with aggressive and severe chronic periodontitis (Kubar *et al.*, 2005). Using serological and molecular approaches, Alexandros Kolokotronis (Aristotle University, Thessaloniki, Greece) demonstrated that the Epstein-Barr virus is associated with chronic periodontitis. Discussion centered on the possible indirect effect of virus infection (local immune suppression that could facilitate bacterial infection) and the possibility of virus re-activation in response to inflammation. William Wade also raised the issue of relevant controls not being identified. The possible use of an antiviral approach to determine if viruses were the causative agents was raised by Deirdre Devine (Leeds Dental Institute, UK).

### Modern Techniques and Their Impact

David Beighton chaired the eighth session with the broad remit to allow presentation and discussion of novel and developing technologies, and how these are contributing to the progression of oral microbiology. A major focus was on further development of molecular techniques for rapid analyses of the diverse flora found at different sites in the oral cavity. Georg Conrads compared data obtained using both traditional culture and a recently marketed microarray detection system for 20 periodontopathogens, the Parocheck 20. This latter system is PCR-based, using 'universal' oligonucleotide primers and the incorporation of fluorescent dyes, followed by hybridization of the sample-mix amplicons to the microarray, which consists of 16S rDNA-based specific oligonucleotides for panel micro-organisms. Rarely have there been directly comparable results between culture and the molecular microarray detection system (Vianna *et al.*, 2005). Bernie Guggenheim did not consider the microarray to be an advancement for oral diagnostics, but it was pointed out that this is the first commercially available system, and that further microarrays encompassing the complete diversity of oral bacteria (culturable and non-culturable) were under development. It was appreciated that this is not a chairside test, but at present is more likely to be useful to the research laboratory. William Wade reminded the audience that PCR was not equally efficient in amplification of all molecules, thus potentially imposing a bias on results. Khalil Boutaga (ACTA, Amsterdam, Netherlands) described attempts at 'universal' real-time PCR amplifications from oral samples, with a formula used against standards to estimate proportions of one species within the mixed sample. He had attempted to validate published 'universal' primer sets and real-time PCR protocols but had been unsuccessful in his studies to determine numbers of *Peptostreptococcus micros* and *P. gingivalis* in samples, and had not obtained correlation with cultural studies. Comments from the floor, including from William Wade and Georg Conrads, suggested that even the best 'universal' primers may be useful for only around 65% of bacteria. Bernie Guggenheim described the use of fluorescently-labeled oligonucleotide probes for FISH analysis using confocal scanning laser microscopy of a six-species biofilm model (Thurnheer *et al.*, 2004). Limitations were imposed by the number of differentially fluorescent dyes available, and by potential problems with cell permeabilization and hybridization stringency for different probes for simultaneous detection of more than one organism within the biofilm. This study had been successful with visual detection of up to three bacterial species at the same time. Global gene expression was introduced by Georg Conrads, with a description of a microarray consisting of specific 50-mer oligonucleotides for each of the 1963 open reading frames in the *S. mutans* UA159 genome. This may be available free for colleagues, although it was pointed out that a rival microarray consisting of 60-mer oligonucleotides was available by application through the NIDCR. Proteomic analysis of *F. nucleatum* membrane proteins was presented by Seyi Dawodu (King's College, London, UK). Seyi described her preparative technique for enrichment of fusobacterial proteins, her particular interest being in efflux proteins. Three genome sequences are available for different *F. nucleatum* subspecies, thus aiding in the identification of purified, or trypsin-digested, proteins following downstream isolation and analyses.

### Virulent Clones and Their Identification

This session was chaired by Mogens Kilian, who introduced general concepts of clonality. Genetic diversity within a species can be subdivided into the core genome for that species, and the flexible gene pool. Around one-quarter of the genes in a genome differ within a species. Clonality is an accumulation of mutations resulting in distinct lineages, some of which are

virulent and some not, and where there is linkage disequilibrium of alleles. An example of an oral species exhibiting clonality is *A. actinomycetemcomitans*. Panmictic clones are a result of recombination where there is random, or close to random, association of alleles. An epidemic generally is a panmictic clone that is successful, and spreads so that it becomes distributed to a wide geographical area. Obvious virulence determinants, e.g., *A. actinomycetemcomitans* leukotoxin, may be good markers of virulence, but other factors may be equally (or more) important, even within leukotoxic strains. Mobile genetic elements such as plasmids, transposons, or bacteriophage may have an impact upon clonality and virulence. Mogens emphasized that, in the study of clonality and virulence, the correct experimental methods must be used. Thuy Do (King's College, London, UK) provided information on a Multi-Locus Sequence Typing (MLST) approach to investigating clonality in *S. oralis/mitis* strains. MLST assigns alleles based on polymorphisms within housekeeping genes. An advantage of MLST over other typing systems is that it standardizes sequence, and hence polymorphism data are produced which are easily exchanged electronically between groups. Housekeeping genes were amplified with oligonucleotide primers utilized in MLST analyses of pneumococci. Preliminary data from several housekeeping genes indicated that *S. oralis* isolates were particularly diverse in comparison with pneumococci. Mike Curtis enquired whether the location of housekeeping genes within a genome had an impact on MLST data, and Mogens Kilian replied that he was aware that clustering of genes within one region of the genome could be a problem, as has been shown in some neisserial studies. Four presentations then focused on confirmed and putative virulence factors in periodontopathogens. Dorte Haubek (Aarhus University, Denmark) described studies using ribotyping, 16S rRNA sequence analysis, and the multicopy nature of rRNA operons and IS650 elements within the genome to measure intra-genomic recombination within *A. actinomycetemcomitans*, particularly relating to the JP2 clone, which is characterized by a 530-bp deletion in the promoter of the leukotoxin gene and is highly leukotoxic. Rolf Claesson (Umeå University, Sweden) showed that highly leukotoxic *A. actinomycetemcomitans* strains with a fully intact promoter region existed. Mike Curtis detailed investigations into the *rag* locus of *P. gingivalis*, originally identified as *ragA*, predicted to encode a *tonB*-dependent receptor protein upstream of *ragB*, encoding a major outer membrane antigen. This locus has a lower GC content than the *P. gingivalis* genome backbone, suggesting horizontal acquisition, and is found in only around 10% of strains, including W50 (Hall *et al.*, 2005). However, 85% of strains express Rag-like proteins from divergent alleles of *ragAB*, and current understanding is that there are four different *rag* alleles. Antibodies raised against one RagB do not cross-react with proteins from strains expressing other *rag* alleles. The allelic type found in W50, and now designated *rag-1*, appears most commonly associated with *P. gingivalis* strains from aggressive periodontal disease. Wil van der Reiden described a study which attempted to correlate the *prtH* gene of *Tannerella forsythensis* with periodontal disease, but had been unable to do so. William Wade made the point that 16S rDNA-directed PCR detection of this particular species in oral samples may not be discriminatory, and that false-positives may be obtained. Juerg Mayer (Basel University, Switzerland) provided information on the *A. actinomycetemcomitans* bacteriophage Aaφ23. It was found in 16/40 of *A. actinomycetemcomitans* strains, with a DNA sequence of 43,033 bp encoding 66 potential reading frames (Resch *et al.*, 2004). There are many similarities in genome organization and function with other lambdoid bacteriophages. Among the advantages for the host bacterium in carrying this bacteriophage may be transduction of antibiotic resistance genes.

## Microbial Virulence Mechanisms

The session on microbial virulence mechanisms, chaired by Mike Curtis, included presentations on the mechanism of action of virulence factors produced by oral spirochetes and *Streptococcus intermedius*. Mike introduced the session with reference to periodontal disease and emphasized that microbial virulence is fundamentally reliant on an understanding of the relative susceptibility of the colonized host (Page and Kornman, 1997). An overview of the potential impact of microbial genomics on virulence research—to allow for the study of genetic variability, transcriptome analysis, and proteomics—was given (Ochman *et al.*, 2000). With reference to a study making a linear genomic comparison of *Bordetella pertussis*, *parapertussis*, and *bronchioseptica*, whereby much genome re-arrangement is apparent, it was pointed out that gene loss and inactivation led to the differences observed (Preston *et al.*, 2004). With reference to the European Microbiology Community, the prospects for the performing of microarray transcriptome analysis of mixed microbial populations, and the genome sequencing of an increased number of oral bacteria, were debated. William Wade stated that proposals were in place for the partial sequencing of 20+ oral species. Dave Dymock (Bristol Dental School, UK) then presented results from a study of the interactions of treponemes with host proteins, with reference to the major sheath protein (Msp) (Edwards *et al.*, 2005) and chymotrypsin-like protease (CTLP) complex as putative virulence determinants. It was shown that *T. denticola* has specificity for binding to the A $\alpha$  chain of fibrinogen. The major fibrinogen adhesin was identified—by affinity chromatography, heat treatment, zymography, and a CTLP mutant—as the CTLP complex. CTLP was also shown possibly to modify proteins on the surface of *T. denticola*, to allow for binding to fibronectin. Mike Curtis asked if fibrinogen and fibronectin were target proteins for proteolytic digestion, and if CTLP was found in *T. pallidum*. Dave confirmed that the CTLP complex proteolytically degraded these proteins. Genes encoding proteins within the CTLP complex have been reported from many different treponemes. With reference to the intermedilysin (ILY) produced by *Streptococcus intermedius*, Rob Whiley (Queen Mary, UK) presented the results from several studies. These included the use of pure ILY and an *S. intermedius* mutant in experiments with human neutrophils, and studies on the invasion of hepatoma HepG2 cells by *S. intermedius*. Howard Jenkinson commented on the potency and functional differences between pneumolysin and intermedilysin. David Beighton asked if a link existed between the levels of hydrolytic enzymes and ILY. In reply, it was stated that enzyme levels are similar across strains, and all strains tested express ILY, although higher levels of ILY are expressed by isolates from deep-seated abscesses in comparison with those from dental plaque.

## Antimicrobial Host Defense Peptides

Deirdre Devine chaired the session on antimicrobial host defense peptides. She reviewed the possible role of host defense peptides during innate and adaptive immune responses in disease and health, as diagnostic aids and as therapeutic agents. Vaishali Rane (Leeds Dental Institute, UK) presented a study entitled 'Detection of antimicrobial host defense peptides in plaque biofilms growing along the gum line'. It was demonstrated, by immunohistochemistry, that HBD1, LL-37, and histatins are able to penetrate plaque biofilms. It was proposed that their distribution may reflect interaction with specific groups of micro-organisms. Discussion centered on the detection of such peptides in the pellicle, whereby they may have a greater influence and interact with other molecules. Rob Allaker then presented

findings on the possible antimicrobial role of the multifunctional peptide adrenomedullin within the oral cavity (Allaker and Kapas, 2003). First, he described possible means by which *P. gingivalis* may resist the action of adrenomedullin. Second, he introduced the concept that post-secretory processing may generate multiple adrenomedullin congeners with enhanced antimicrobial activity. Deirdre asked if the peptide fragments were able to resist proteolytic digestion by *P. gingivalis*. Rob commented that no difference was observed between parent and protease-deficient mutant strains. Discussion included how to determine the relative importance of individual host defense peptides in gene knockout mice or siRNAs. Howard Jenkinson suggested that such peptides may be of particular importance in newborns, and were likely to have an impact on how an individual acquires a 'normal' microflora.

## Modulation of Host Defenses

This session, also chaired by Mike Curtis, focused on how *A. actinomycetemcomitans* and *P. gingivalis* modulate host defenses, an important virulence characteristic of these periodontopathogens. Anders Johansson (Umeå University, Sweden) demonstrated that human macrophages were very sensitive to low concentrations of *A. actinomycetemcomitans* leukotoxin. IL-1 $\beta$  production and secretion were significantly induced, but this was not observed for IL-6 and TNF- $\alpha$ . Secretion of bioactive IL-1 $\beta$  requires activation of caspase 1 (Kelk *et al.*, 2005). This would potentially enhance and confuse the immune response. Mike enquired whether monocytes were present in tissues to which *A. actinomycetemcomitans* would have access. This must be so, because there is a high antibody response, in particular to the leukotoxin. It appears that antibodies are protective in tissue, but not necessarily in the periodontal pocket, since it takes several weeks for an antibody response. Sotos Kalfas (Aristotle University, Thessaloniki, Greece) posed the question of what is unique about *A. actinomycetemcomitans* that results in detection or isolation as the sole micro-organism within some periodontal pockets. He had studied the effects of *A. actinomycetemcomitans* cytolethal distending toxin (CDT) on gingival fibroblasts and periodontal ligament cells. He described how the induction of Receptor Activator of NF $\kappa$ B ligand (RANKL) resulted in increased osteoclastogenesis. However, stimulation of RANKL was independent of anti-inflammatory mediators, a result confirmed with the use of a CDT-negative mutant (Belibasakis *et al.*, 2005). Thus, the two *A. actinomycetemcomitans* toxins appear to have different mechanisms for modulation of host defenses. This theme also included two presentations on *P. gingivalis*, with George Hajishengallis (Louisiana State University, New Orleans, USA) speaking on inflammatory interactions with this organism *via* toll-like receptors. Fimbriated *P. gingivalis* cells induce a higher pro-inflammatory response in macrophages than do non-fimbriated cells. George presented data indicating evidence for a novel TLR2-dependent signaling pathway that leads to CD11b-CD18 activation in human monocytes or neutrophils upon recognition of *P. gingivalis* fimbriae through CD14 (Harokopakis and Hajishengallis, 2005). TLR-2 may therefore be acting as a signaling receptor, and further understanding of this mechanism may lead to novel methods of controlling infection-driven chronic inflammatory conditions, such as periodontal disease. Nobuhiro Takahashi (Tohoku University, Japan) showed that *P. gingivalis* LPS-induced IL-8 production is inhibited by the arginine-specific cysteine protease, Rgp. The mechanism for this inhibition is through proteolytic degradation of CD14 on the surfaces of human gingival fibroblasts (Tada *et al.*, 2002).

## Host Responses

The final session, on host responses, commenced with a presentation by Bertil Kinnby (Malmö University, Sweden) on plasminogen-binding oral bacteria. It was concluded that strains of streptococci from inflammatory lesions displayed higher capacity than strains from plaque, and may be an indicator of potential virulence and initiation of tissue degradation. Danaï Apatzidou (Aristotle University, Thessaloniki, Greece) then described a study to examine a possible relationship among PCR detection of *T. denticola*, serum antibody and avidity to this organism, and clinical findings over a six-month period following periodontitis therapy. Deirdre Devine commented on the unchanged antibody response over time in relation to very low detection of *T. denticola*. The final presentation of the workshop was given by Marja Laine (ACTA, Amsterdam, Netherlands) on pattern recognition receptor gene polymorphisms in periodontitis. Of particular interest was the CD14 T/T genotype and its association with severe disease. It was hypothesized that in this particular genotype, the expression of the CD14 receptor was increased, leading, in turn, to a potential up-regulation of pro-inflammatory cytokine levels.

On the afternoon of the final day, delegates were taken by boat for an excursion to view the monasteries at Mount Athos on the easternmost Halkidiki peninsula. Back on dry land, dinner was then served at the Sousourada Restaurant in Athytos Village. After a vote of thanks to Sotos Kalfas and his organizing committee, it was announced that the next European Oral Microbiology Workshop will be held in Finland, in 2008, and will be organized by Eija Könönen and her colleagues.

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