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MEETING REVIEW

Genetics of Streptococci, Lactococci, and Enterococci: Review of the Sixth International Conference

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The Sixth International Conference on Streptococcal, Lactococcal, and Enterococcal Genetics, sponsored by the American Society for Microbiology, convened 14 to 17 April, 2002, in Asheville, N.C. From its inception in 1981, this conference has highlighted the best science in the field and has provided a forum for young investigators to present their work, often for the first time, to an international audience. This year's conference continued both traditions, with 39 outstanding presentations from established and young investigators alike, along with 150 poster presentations from among the more than 250 attendees of the meeting. Many of the contributions to the meeting are highlighted in this review, and a number of related articles appear in this issue of the *Journal of Bacteriology* (1, 5, 10, 13, 16–18, 24, 25).

The streptococci have played a central role in the development of bacterial genetics, beginning with experiments described by Griffith in 1928, in which he provided the first demonstration of bacterial gene transfer (12). Those experiments, involving *Streptococcus pneumoniae*, were the first description of genetic transformation in bacteria and led to the discovery in 1944 by Avery, MacLeod, and McCarty that DNA was the "transforming principle" of Griffith's experiments and hence the genetic material (2). Since the first streptococcal-genetics meeting, the lactococci and enterococci have been designated as separate genera; antibiotic resistance has increased from an emerging observation to a worldwide health issue; historic observations such as Griffith's capsule transformation have been described at the molecular genetic level; and the gene transfer systems, plasmids, phage, and transposons that first brought streptococcal researchers together now provide the tools for dissecting metabolic and virulence pathways, while still maintaining their own allure. The present time represents another significant era in the history of the streptococci, as we witness the completion of the genome sequences of many streptococcal, lactococcal, and enterococcal species (3, 4, 8, 11a, 14, 26, 27a, 28) and begin the transcriptional and proteomic analyses that will lead us to an unprecedented understanding of the regulatory events that take place within the cell.

The opening lecture by Claire Fraser (The Institute for Genomic Research, Rockville, Md.) provided an overview of the genomic revolution and set the scene for a number of presentations on genomes of *Streptococcus*, *Enterococcus*, and *Lactococcus* species. The next frontiers, made possible by newly developed technologies, will be the discovery and analyses of genomes of yet-to-be-cultured microbes and the genomic analyses of microbes colonizing the oral cavity, respiratory tract, and intestinal system of the human body. Moreover, with the increasing number of known genome sequences and the exploding number to come, comparative genomic analysis will be a powerful tool with which to monitor microbial evolution and will provide insights into the multiple processes that shape microbial diversity. A special case that may become a paradigm in this area is the comparative sequence analysis of seven strains of *Bacillus anthracis*. The chromosomal sequence comparison of two related strains revealed the presence of only two short inserted or deleted sequences (indels) and two single-nucleotide polymorphisms (SNPs) in their 5.2-Mb chromosomes, while a few dozen more SNPs were found on each of the two plasmids (23). This detailed genomic analysis allowed for an unprecedented distinction at the strain level, revealing a powerful tool for investigating infectious-disease outbreaks. Other medical applications relevant for the pathogenic streptococci involve the discovery of new drug targets and the identification of candidates for vaccine production. However, the challenges ahead deal with all areas of microbiology and include analysis of gene expression under natural conditions, identification of regulatory networks, and expansion and continuous curating of microbial databases, for which there is an urgent need.

FUNCTIONAL AND COMPARATIVE GENOMICS WITH A VIEW TO EVOLUTION

The meeting was dominated by new genome sequencing results, particularly comparative genomic analyses of streptococci and other gram-positive genera, both commensals and pathogens. Joseph Ferretti (University of Oklahoma Health Sciences Center) presented the genomes of *Streptococcus pyogenes* (group A streptococcus) and *Streptococcus mutans*, two pathogenic streptococci that occupy different niches in the

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human host. The *S. pyogenes* M1 rheumatogenic strain SF370 and the M49 nephritogenic strain NZ131 have been completely sequenced, and partial genome sequences have been obtained for four other strains (representing an M3, an M5, and an M18 strain as well as another M49 strain). The completed M1 and M49 strains varied much more than the sequenced *B. anthracis* isolates and showed a minimum of 18,000 SNPs in their respective genomes of 1,852 and 1,771 kb. Further comparisons indicated that among the 1,696 predicted protein-encoding genes in NZ131, 1,063 genes are virtually identical to genes in the sequence of SF370, 452 share some significant degree of sequence similarity, and 181 genes are not found in SF370. The analyses of these two *S. pyogenes* genomes allowed for identification of at least 10 copies of insertion elements (IS elements) and many new virulence factors, some of which may be encoded by the prophages that are present in the M1 (3 complete copies and 1 incomplete copy) and M49 (1 complete copy and 2 incomplete copies) strains. Remarkably, the 2,030-kb genome of *S. mutans* UA159, a serotype c isolate associated with dental caries, had no prophage but contained 12 copies of IS elements and 2 large transposons. The overall comparison of the predicted genes of *S. mutans* with those of other streptococci indicates the highest degree of similarity with *S. pneumoniae* (67%) and *S. pyogenes* (64%), testifying to the close evolutionary relationship of these highly AT rich gram-positive bacteria.

Streptococcus agalactiae (group B streptococcus) normally colonizes the gastrointestinal and urogenital tracts and can act as an opportunistic pathogen in compromised individuals. The organism is a leading cause of invasive neonatal infections (septicemia, meningitis, and pneumonia). The 2.2-Mb genome of the serotype III strain NEM316 revealed a number of interesting features, especially when compared to the *S. pyogenes* genome. Philippe Glaser (Institut Pasteur, Paris, France) noted that there were no complete prophages in *S. agalactiae* but observed 8 putative IS elements, a large number of transposons, and several putative integrases in 13 "mobility islands," suggesting that acquisition of mobile genetic elements played an important role in the development of virulence. For example, two known virulence factors, one encoding a laminin binding protein and one encoding a peptidase, were flanked by defective IS elements. Of 29 surface proteins encoding LPXTG motifs, 17 were located on mobility islands. Comparisons between the genomes revealed a significant conservation of gene order between *S. pyogenes* and *S. agalactiae*. In contrast, almost no synteny was observed between *S. agalactiae* and *S. pneumoniae*, and those differences in gene order were attributed to the high recombination frequency of the naturally competent *S. pneumoniae*.

Analysis of two *S. pneumoniae* genomes by Susan Hollingshead (University of Alabama at Birmingham) revealed that plasticity zones were responsible for many interesting features of this pathogen, which causes more than 1 million deaths globally, mainly in young children and the elderly. The plasticity zones represented as much as 10% of the genome and encoded pathways for capsular biosynthesis, bacteriocin production, and surface protein modulation, traits that are of adaptive significance. With atypical GC content, the plasticity zones were likely acquired by horizontal gene transfer and appear to be responsible for the lack of genome synteny with other pathogenic streptococci. Interesting features in the *S. pneumoniae* genome included 69 putative cell surface proteins and an unusual gene encoding a cell wall-anchored protein of more than 4,000 amino acids composed mostly of a repeated serine-alanine-serine-alanine (SASA) motif. It was noted that this gene was adjacent to seven genes encoding glycosyltransferases, and it was suggested that the enzymes might act on the serine in SASA motifs to create O-linked glycosylations and produce a "mucin-like" glycopeptide. As with the other streptococcal genomes analyzed, a large number of carbohydrate transporters were also identified, reemphasizing the importance of sugar utilization for metabolic activity and survival.

The first enterococcus genome sequence was completed for *Enterococcus faecalis*, an opportunistic pathogen that is the leading cause of life-threatening nosocomial infections in the United States. Linda Bannerjei (The Institute for Genomic Research) described the 3.2-Mb genome and three plasmids, one of which contains a conjugative transposon encoding vancomycin resistance. Genome analysis revealed that both antibiotic resistance and virulence determinants were encoded by mobile genetic elements that could contribute to the spread of these features to other nosocomial gram-positive pathogens. The importance of gene transfer and acquisition was further emphasized by the observation that 10% of the predicted open reading frames were prophage related. Integrases were positioned near genomic islands encoding virulence factors found in other gram-positive pathogens, including *Listeria monocytogenes*, *Staphylococcus aureus*, *S. pneumoniae,* and *Streptococcus sanguis*. Other interesting features included 35 phosphoenolpyruvate transport systems for efficient carbohydrate utilization and 17 two-component signal transduction systems for environmental sensing and response. Genetic analysis revealed a commensal organism with substantial potential to acquire and transmit virulence factors and antibiotic resistance determinants.

The relatedness of the streptococcal, lactococcal, and enterococcal genomes, despite the diverse habitats of the organisms, was a theme that echoed throughout the meeting. Dusko Ehrlich (Institut National de la Recherche Agronomique, Jouy en Josas, France) described the genomes of two lactic acid bacteria that share the streptococcal group N antigen and are involved in industrial dairy fermentations, viz., the yogurt bacterium *Streptococcus thermophilus* (strain CNRZ 1066; 1,796 kb) and the cheese starter *Lactococcus lactis* (strain IL-1403; 2,365 kb). Both genomes are highly flexible; they contain 42 and 56 copies of IS elements and 1 and 5 prophages, respectively. A peculiar feature of the genome of *S. thermophilus* is the presence of many (at least 229) truncated genes that may account for more than 10% of the total set of predicted genes. Comparison of the genomes of these two dairy species with those of the related pathogens *S. pneumoniae* and *S. pyogenes* allowed for the identification of 746 common genes. In addition, 36 genes, several of which were involved in N metabolism, were found only in the food-related bacteria, while 44 putative pathogen-specific genes were identified only in the virulent streptococci. Many of the latter were involved in carbohydrate utilization. By analyzing the conservation in gene order among these related species, a remarkable genomic colinearity was observed that suggested the presence of symmetric chromosome inversion.

Genomic analyses of the streptococci have been accompanied by genomic analyses of their bacteriophages, both virulent and temperate (7). Through comparison of numerous genomes from streptococcal and lactococcal phages, Harold Brüssow (Nestle Research Center, Lausanne, Switzerland) revealed a conserved genome structure composed of four major modules: late genes (head, tail, encapsidation), tail fiber and lysis-lysogeny, replication, and transcriptional regulation. It was noted that sequence variation in bacteriophages occurs most often in the modules harboring the tail fiber region, affecting variable host range, and the lysogenic sequence responsible for prophage integration and maintenance. Prophage sequences have been found throughout the streptococci, revealing both complete (active) and partial (remnant) elements. For example, *S. pyogenes* SF370 harbors eight prophage elements: one complete and inducible, five with massive deletions, and two carrying mutations in key phage genes. Residing prophage elements appear to undergo deletions and mutations at high frequencies, reflecting a propensity for the bacterium to remove this extra, foreign DNA. Interestingly, phage remnants often contain genes of the lysogenic and replication modules, which may exhibit constitutive levels of transcription over regions associated with maintenance of lysogenic functions. In *S. thermophilus*, this activity over a repressor likely provides immunity against superinfection by related phages present in a dairy fermentation plant. In *S. pyogenes*, this transcribed region encodes superantigens, toxins, and DNases that may contribute to the virulence of the host. It was suggested by Brüssow that, in this capacity, prophages and prophage remnants can be responsible for lysogenic conversions that contribute foreign genes and thereby increase the competitiveness of the lysogen in its ecological niche.

Genetics of streptococcal populations have been analyzed by using multilocus sequence typing (MLST). Mark Enright (University of Bath, Bath, United Kingdom) described MLST analyses in which the sequence of an internal 450-bp region from each of seven housekeeping genes is compared among a large number of isolates to yield a sequence type (ST). Studies of *S. pyogenes* demonstrated a strong association between the *emm* sequence type (encoding the M protein) and the ST in some strains but high diversity in other strains. Likewise, in *S. pneumoniae*, restricted genetic diversity occurs among strains of some capsular serotypes but not others. Consistent with the results from genome sequencing, these types of analyses suggest that gene transfer and recombination occur at high frequency in the streptococci and play a significant role in the evolution of these organisms.

CELL-TO-CELL SIGNALING AND GENE TRANSFER: NEW IDEAS ABOUT OLD PHENOMENA

Horizontal gene transfer is considered to be one of the important factors in microbial evolution and the spread of virulence factors in pathogenic bacteria (21). Various streptococci, enterococci, and lactococci have become paradigms for studying the mechanisms of cell-to-cell signaling and gene transfer. In *S. pneumoniae* and *S. mutans*, transformation involves a quorum-sensing mechanism in which a competence-stimulating peptide (encoded by *comC*) induces competence via a two-component sensor histidine kinase (*comD*) and cognate response regulator (*comE*) system (6). The potential for a similar means of gene transfer in other streptococci is suggested by the finding of competence genes in many of the genome sequences, including those of *S. pyogenes, S. agalactiae*, and *L. lactis*.

S. mutans lives in a biofilm environment in the host, and under these conditions, the cells are perpetually competent and exhibit a 10-fold-higher transformation efficiency than competent cells grown in planktonic culture. Dennis Cvitkovitch (University of Toronto, Toronto, Canada) described the *S. mutans* system and presented the functional analysis of a new two-component regulatory system (consisting of the tandem *hk11* and *rr11* genes) involved in the formation of biofilms and survival at an acidic pH. Both functions are considered to be virulence factors in dental caries, and it is speculated that their regulation via the *hk11*/*rr11* system is dependent on quorum sensing involving ComC, the competence-stimulating peptide pheromone. The *hk11*/*rr11* genes are conserved among other AT-rich gram-positive bacteria, including *S. pneumoniae*, which has been a model for competence development and its control by quorum sensing. The early events in this control were discussed by Jean-Pierre Claverys (Centre National de la Recherche Scientifique and University of Toulouse, Toulouse, France), who used an in vitro *mariner* transposon mutagenesis system to generate *S. pneumoniae cup* (competence up) mutants that exhibited elevated expression of *comC*. More than 50 *cup* mutants belonging to 10 classes that included genes involved in lipid, cell wall, and nucleotide metabolism were identified. The results provide evidence for the suggestion that competence in *S. pneumoniae* is a general stress response and that the competence-stimulating peptide, encoded by *comC*, acts as an alarmone. Adaptation to the human host is crucial for a bacterial pathogen and might involve different genetic mechanisms. In the case of *S. pneumoniae*, Jeffrey Weiser (University of Pennsylvania School of Medicine) reported that the genetic instability leading to gene inactivation is important for adaptation to the human respiratory tract. Naturally occurring inactivation of pneumococcal genes results from reversible frameshift mutations, deletions, and oxidative damage of guanine residues caused by the hydrogen peroxide generated by this species when it is grown under aerobic conditions. Under such growth conditions, the pneumococcal mismatch repair system is overwhelmed, leading to a high rate of mutation. Competence, which is down-regulated during anaerobic growth, may therefore be a mechanism for repairing the mutations generated during aerobic metabolism.

Virulence and antibiotic resistance genes are often associated with mobile elements that facilitate their dissemination (11, 19). Barbara Spellerberg (University Hospital Aachen, Aachen, Germany) described a new composite transposon in *S. agalactiae* and provided evidence for its ability to mobilize and be transferred. The transposon is flanked by 2 copies of IS*Sag2*, a member of the IS*3* family, and includes the virulence genes *lmb*, implicated in binding human laminin, and *scpB*, encoding a C5a peptidase. The transposon was present in *S. agalactiae* isolates of human origin but generally absent in bovine isolates. Similarly, homologous genes occurred in human but not animal isolates of *S. pyogenes*, group C streptococci, and group G streptococci, suggesting an important role of the transposon in adaptation of the bacteria to the human host. The presence of

a group II intron within the transposon has been observed in some *S. agalactiae* isolates. These introns are self-splicing, mobile RNAs that integrate into specific target sites in a process referred to as "homing." Joanna Klein (and Gary Dunny, University of Minnesota) analyzed the splicing of these elements in *L. lactis* and identified residues conserved among related introns that were required for efficient splicing. David Mills (University of California, Davis) described methods for using group II introns as tools in generating targeted disruptions and controlling gene regulation in *L. lactis* and other low-AT gram-positive bacteria. Because of the efficient homing mechanism, no antibiotic selection is necessary and the insertions are genetically stable, making them attractive tools for manipulating food grade bacteria.

BACTERIOCINS AND CYTOLYSINS

New roles for bacteriocins, which are small, ribosomally synthesized peptides classically associated with antimicrobial activity, were revealed in studies reported by Nick Heng (University of Otago, Dunedin, New Zealand) and Oscar Kuipers (University of Groningen, Groningen, The Netherlands). Cytolysins with features closely resembling those of bacteriocins and are important in pathogenesis were described by Wolfgang Haas (University of Oklahoma Health Sciences Center), Nathan Shankar (University of Oklahoma Health Sciences Center), and Victor Nizet (University of California, San Diego).

Heng (with John Tagg and G. R. Tompkins) found that inactivation of genes involved in the early stages of transformation in *Streptococcus gordonii* resulted in nontransformable strains that failed to produce two bacteriocins, STH_1 and STH_2. The phenotype could be complemented by an exogenous competence-stimulating peptide, but the inactivation of genes important in DNA uptake, a later stage of transformation, had no effect on bacteriocin production. These molecules thus appear to play an important role in transformation and could potentially kill other bacteria for the purpose of eliminating competing recipient strains and/or releasing their DNA for uptake by the competent bacteriocin producer. Kuipers described two novel, plasmid-encoded bacteriocins, Bac513 and Bac2, from *L. lactis*. LmrB, the immunity protein for both, shares homology with the human multidrug resistance P glycoprotein, functions as an ATP-dependent multidrug transporter, and is involved in secretion of Bac2. In addition, LmrB and P glycoprotein share substrate specificity, further suggesting a fundamental role for these types of transporters in both eukaryotes and prokaryotes.

In *E. faecalis*, the cytolysin subunit precursors are ribosomally synthesized and then posttranslationally modified to resemble the lantibiotic bacteriocins. Following secretion by an ABC transporter, they are cleaved and activated extracellularly, thereby becoming capable of forming pores in bacterial and eukaryotic membranes. Haas found that transcription of the cytolysin operon was repressed by two small proteins encoded upstream and transcribed in the direction opposite to that of the cytolysin operon. Addition of the activated small cytolysin subunit alleviates the repression, and this subunit is important in quorum sensing and autoinduction of cytolysin expression. In contrast, the large cytolysin subunit reduces cytolysin expression, possibly by binding the small subunit and thereby reducing autoinduction. The two small proteins involved in repression of the system are not the typical histidine kinase and response regulator observed in most quorum-sensing systems, suggesting the involvement of a novel mechanism of regulation. Shankar found that the cytolysin and other well-documented virulence factors of *E. faecalis*, including aggregation substance and the surface protein Esp, are located within a 154-kb pathogenicity island. The element is the first clear pathogenicity island to be identified in a gram-positive bacterium. It was shown that the cytolysin operon and the gene encoding Esp can be specifically deleted from this island at a high frequency, suggesting the involvement of a novel and efficient recombination mechanism for modulating bacterial virulence in enterococci.

S. pyogenes and *S. agalactiae* are beta-hemolytic streptococci that lyse erythrocytes due to the synthesis of protein exotoxins. In *S. pyogenes*, the hemolysin streptolysin S (SLS) is encoded by the *sag* locus, which is comprised of nine genes (*sagA* through *sagI*) and displays several features characteristic of a bacteriocin-biosynthetic operon. The first gene, *sagA*, is the structural gene for the SLS toxin, which is distantly related to the enterococcal cytolysin. The last three genes of the operon likely encode an ABC transporter involved in the export of the toxin. Nizet found that SLS contributes to the pathogenesis of streptococcal soft tissue infection in a murine model and that in vitro it elicits direct cytotoxicity and inhibits neutrophil phagocytosis. The hemolysin activity of *S. agalactiae* is due to CylE, which, unlike the bacteriocin-like cytolysins, is a large (78-kDa) protein encoded within a fatty acid biosynthesis operon. Its production is correlated with injury of various epithelial and endothelial cells and macrophages. It stimulates inducible nitric oxide synthase transcription and nitric oxide production in macrophages, can trigger macrophage apoptosis, and is also a key factor in activating endothelial-cell genes implicated in the neutrophilic inflammatory response of *S. agalactiae*-induced meningitis. Nonhemolytic mutants are dramatically less virulent in the neonatal rat model of infection.

GLOBAL REGULATORY CONTROLS

Most cellular functions are under some type of global control. As noted above, induction of competence may represent one such global response. Zezhang Wen (and Robert Burne, University of Florida) reported on the role of the ubiquitous autoinducer II (AI-2) pathway of quorum sensing in gene expression in *S. mutans* and found that a LuxS mutant was no longer transformable. In addition, more than 35 proteins, including DnaK and GroEL, were up-regulated in the mutant and at least 17 others were down-regulated. LuxS of *S. mutans* may, therefore, be involved in the development of competence and in the regulation of the stress response or homeostasis. LuxS homologues have been identified in other streptococci, suggesting a potentially common mechanism of regulation. Jean-San Chia (National Taiwan University, Taipei) used RNA fingerprinting and Northern blot hybridization to characterize genes of *S. mutans* that were induced or repressed by stress. Among the genes that were induced following exposure to high osmolarity or temperature and low pH was that encoding the general stress protein GSP-781, which is ubiquitous in

streptococci, enterococci, and lactococci. Sequence analysis revealed that GSP-781 corresponds to the immunodominant antigen protein IDG-60, a glycosylated protein containing sialic acid, mannose, galactose, and *N*-acetylgalactosamine. Inactivation of the gene encoding IDG-60 revealed that this protein is essential for maintaining the integrity of the cell wall and the uniformity of cell shape and for bacterial survival under stress conditions.

The acid tolerance response is especially important for lactic-acid-generating bacteria, which grow best under neutral pH conditions (22). Beyond DnaK, GroEL, GroES, and ClpP, little is known about the proteins involved in this response in the streptococci. Emmanuelle Maguin (Institut National de la Recherche Agronomique) investigated the acid tolerance response and its regulation in *L. lactis*. Like those of *S. pneumoniae*, *S. pyogenes*, and *E. faecalis*, the *L. lactis* genome lacks a general stress response regulator homologous to σ^B. Analysis by transposon mutagenesis revealed that phosphate limitation in a *pst* mutant induced a high tolerance to acid and oxidative stress, suggesting that the phosphate-controlled regulon may play a role in acid stress adaptation. Similarly, alterations of the intracellular guanine nucleotide pools (*guaA* and *relA* mutants) increased acid and other stress tolerances and resulted in cell wall alterations. Proteomic analysis led to the proposal that the lactococcal acid tolerance response proteins belong to the guanine pool and phosphate stimulons.

Bacteria interact with host cells through the action of global regulators responding to environmental signals, host factors, and other bacterial signals. Bernd Kreikemeyer (and Andreas Podbielski, University Hospital Rostock, Rostock, Germany) described a number of the complex global regulatory pathways operating in *S. pyogenes*. Three response regulators (Mga, RofA, and Rgg) without cognate sensors are known to positively regulate the expression of various sets of virulence genes. These regulatory pathways are linked: RofA negatively regulates Mga, whereas Rgg controls several regulatory pathways including Mga and the two-component regulatory system CovR/CovS, which regulates the Has operon involved in capsule production. Rgg also regulates the expression of the FasBCAX system, which displays similarity to the quorum-sensing regulatory systems Com and Agr from *S. pneumoniae* and *S. aureus*, respectively. Fas induces the expression of a number of proteins, including SLS, which in turn may influence the expression of other virulence factors. Using microarray analyses, James Musser (National Institutes of Health Rocky Mountain Laboratories, Hamilton, Montana) found that expression of more than 200 genes was altered in *rgg* mutants of *S. pyogenes*. These overlapping regulatory pathways thus enable a fine-tuning of the virulence genes to properly manage bacterium-host cell interactions, bacterial virulence phase variation, and intracellular persistence. Musser also noted that genomic analyses have opened the way for the rapid identification and characterization of potential virulence factors and vaccine candidates. From the genome sequences of four *S. pyogenes* strains, four conserved sequences encoding previously unknown putative surface proteins have been identified. Western blot analyses using patient sera demonstrated expression of the proteins in vivo, and antibodies to one of the proteins were protective in mouse challenge models.

The molecular mechanisms by which the pleiotropic regulator Mga of *S. pyogenes* controls virulence gene expression, as well as its own expression in response to environmental signals, were investigated by Kevin McIver (University of Texas Southwestern Medical Center, Dallas). Of four helix-turn-helix domains present in the NH_2 moiety of Mga, two were found to be necessary for Mga activity in vivo. One of these domains is not required for DNA binding in vitro. However, in vivo, this domain is required for *mga* autoactivation but not for the activation of the other regulon genes. Thus, it was concluded that Mga contains two DNA binding domains possessing complementary activities in vivo. Because Mga is not part of a typical sensor kinase-response regulator pair, additional factors are expected to be involved in the regulatory pathway. Consistent with this hypothesis, putative membrane proteins with potential roles in controlling *mga* expression were identified through transposon mutagenesis.

Lipoproteins are major components of the gram-positive cell envelope that exhibit enzymatic, solute binding, and protein export functions (27). Dean Harrington (University of Bradford, Bradford, United Kingdom) discussed work in which he, Andrea Hamilton, and Iain Sutcliffe (University of Sunderland, Sunderland, United Kingdom) examined lipoprotein biosynthesis in *Streptococcus equi*, the causative agent of strangles, a highly infectious airway disease that represents 30% of all equine infectious diseases. Lipoproteins were found to constitute approximately 1.5% of the streptococcal proteome. Inactivation of diacylglycerol transferase (Lgt), the enzyme necessary for lipidation of proteins, resulted in the loss of a large number of *S. equi* lipoproteins. Inhibition of Lgt activity may thus have pleiotropic effects that could influence the virulence of the organism, and disruption of lipoprotein biosynthesis may serve as a potential drug target in this, and possibly other, streptococcal diseases. Among the lipoproteins lost in the Lgt mutant was MBL, a putative metal binding protein with homology to ScaA of *S. gordonii*. The ScaCBA metal transporter system was further described by Nick Jakubovics (and Howard Jenkinson, University of Bristol) in relation to its role in Mn^{2+} homeostasis in *S. gordonii*. ScaA, the lipoprotein component of the system, is up-regulated by low Mn^{2+} concentrations and repressed by high Mn^{2+} concentrations but is not similarly affected by other metal ions. Its transcription is regulated by the metallorepressor protein ScaR, which has homologues in many streptococcal, lactococcal, and enterococcal species. Sca permease mutants require elevated levels of Mn^{2+} to grow anaerobically and are unable to grow under aerobic conditions. Although they are able to adhere to surfaces, the mutants are unable to form biofilms. The influence of Mn^{2+} on oxidative stress was further observed in relation to superoxide dismutase activity, which was reduced by low Mn^{2+} concentrations. The low levels of manganese present in tissues, its essentiality for the growth of many lactic acid bacteria, and the occurrence and conservation of multiple Mn^{2+} transport systems among bacteria highlight the importance of these systems in bacterial survival and virulence and suggest another potential strategy for antimicrobial targeting.

SURFACE STRUCTURES

The characterization of cell surface structures received significant attention at the Sixth International Conference. A

number of reports revealed the presence of glycolytic proteins on the cell surface. Joanna Wilkins (GKT Dental Institute, London, United Kingdom) described studies in which she, Karen Homer, and David Beighton examined the cell surface-associated proteins expressed by *Streptococcus oralis* at pH 7 versus pH 5.2. Twenty proteins were identified by two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectroscopy. Predominant among these were glyceraldehyde-3-phosphate dehydrogenase, enolase, fructose-bisphosphate aldolase, and a lipoprotein. Differentially expressed proteins were superoxide dismutase and the protein translation factors Tu and G. These observations significantly extend our view of the types of cell surface proteins that are found on streptococci and raise many interesting questions as to the potential extracellular roles of these "cytoplasmic" enzymes.

Many surface proteins in gram-positive bacteria are anchored to the cell surface by the action of the sortase, which covalently links proteins containing an LPXTG motif to the cell wall (20). Genome analyses revealed sortase homologues in most of the streptococcal species. Tim Barnett (and June Scott, Emory University, Atlanta, Ga.) reported that *S. pyogenes* encodes at least two sortases, SrtA and SrtB. While *srtA* was detected in all *S. pyogenes* strains tested (representing multiple M-protein serotypes) and in group C and group G streptococci, *srtB* was present in only a limited number of *S. pyogenes* isolates. Functional studies of *S. pyogenes* revealed that the two enzymes are required for anchoring different subsets of proteins to the cell wall. The M6 protein, protein F, and ScpA were among those requiring SrtA, whereas T6 is sorted by SrtB.

Antigenic variation of surface structures represents an important mechanism by which pathogens can avoid host defenses. In *S. pyogenes*, the surface-localized M proteins have an N-terminal hypervariable region (HVR) of ~50 amino acid residues that binds the human complement inhibitor C4BP and is essential for the antiphagocytic activity of this protein. Gunnar Lindahl (Lund University, Lund, Sweden) found that synthetic peptides containing isolated HVRs are capable of highly specific binding to the same site in C4BP, despite essentially no amino acid identity and no immunological cross-reactivity between the HVRs. Loss of C4BP binding ability, due either to mutations in the HVR or blocking by antibodies to HVR, enhances phagocytosis. The results provide new insights into the mechanism of phagocytosis resistance and suggest that the bacteria are able to avoid phagocytosis by binding a host ligand that prevents complement deposition on the cell surface or impedes some later step in the process. Not all HVRs bind C4BP, and the binding of other host ligands may also be important in the pathogenesis of *S. pyogenes*.

In recent years, genetic analyses have brought forth renewed interest in the fundamental surface structures and exopolymers of the streptococci. Knowledge of the genes involved in peptidoglycan, teichoic acid, capsule, and exopolysaccharide biosynthesis has led to a new appreciation of the mechanisms of synthesis and the functions of these complex polymers. Most gram-positive bacteria produce branched peptidoglycan precursors resulting from the transfer of various L-amino acids or glycine to the εNH_2 group of the L-Lys_3 of the pentapeptide. Michel Arthur (INSERM EMI 0004, Université Paris VI, Paris, France) reported that three *E. faecalis* enzymes are necessary and sufficient for tRNA-dependent addition of two L-Ala residues to the UDP-MurNAc pentapeptide: AlaS is the alanyl-tRNA synthase, and BppA1 and BppA2 are the ligases specific for the incorporation of the first and second L-Ala's of the side chain, respectively. In *E. faecalis*, *bppA1* appears to be essential whereas *bppA2* can be deleted, leading to production of peptidoglycan precursors with only one L-Ala residue. *E. faecalis bppA2* mutants display a higher susceptibility to penicillin due to the inability of the D,D-carboxypeptidase PBP5 to process the altered side chain of the pentapeptide stem.

In *S. agalactiae,* disruption of *ponA*, which is predicted to encode a bifunctional, high-molecular-weight penicillin binding protein (PBP1a homologue) with transglycosylase and transpeptidase activities in peptidoglycan synthesis, resulted in reduced virulence in a neonatal rat sepsis model. Amanda Jones (and Craig Rubens, Children's Hospital and Regional Medical Center, Seattle, Wash.) found that the *ponA* mutants were as sensitive to opsonophagocytic killing as capsule-negative mutants, although there was no evidence for alterations in the capsular polysaccharide. Decreased virulence of PBP1a mutants has been reported for other gram-positive bacteria, but these findings are the first to identify a possible mechanism for the role of PBP1a in pathogenesis and to identify a noncapsular component of *S. agalactiae* that is important in resistance to opsonophagocytosis.

Lipoteichoic acids (LTAs) are membrane-anchored surface polymers unique to gram-positive bacteria (20). Ion binding, membrane stabilization, anchoring of proteins to the bacterial surface, and interactions with host cells are functions that have been attributed to these polymers. Analysis of the esterification of the LTA of *S. agalactiae* with D-Ala residues was presented by Claire Poyart (INSERM U570, Université Paris V, Paris, France). This reaction is catalyzed by products encoded by the *dlt* operon, which is comprised of four genes (*dltA* through *dltD*) necessary for formation of the D-Ala ester of LTA and two genes (*dltRS*) that encode a two-component regulatory system. In this bacterium, the *dlt* operon is up-regulated through DltR/DltS when the amount of D-Ala incorporated in the LTAs decreases. A DltA mutant that lacks D-Ala-substituted LTA displayed increased susceptibility to cationic peptides, including human defensin, and was more susceptible to killing by murine macrophages and human neutrophils. After systemic infection, this mutant was eliminated more rapidly from murine organs (brain, liver, and spleen) and from the blood.

In gram-positive bacteria, polysaccharides can be associated with the cell surface as capsular polysaccharides (CPS) (e.g., *S. agalactiae, S. pneumoniae, S. pyogenes*) or secreted as exopolysaccharides (EPS) into the environment of the cell (*S. thermophilus, L. lactis*). The gene clusters involved in EPS biosynthesis are similar to those involved in CPS biosynthesis. Michiel Kleerebezem (Wageningen Centre for Food Science, Wageningen, The Netherlands) reported that EPS biosynthesis in *L. lactis* NIZO B40 depends on both plasmid-borne (*eps*) and chromosome-borne (*galU*, *galE*, and *rfbACBD*) genes and that the latter are involved in synthesis of primary EPS precursors (glucose-1-P→UDP-glucose, UDP-glucose→UDP-galactose, and glucose-1-P→dTDP-rhamnose, respectively). Disruption and overexpression analyses of these genes provided

evidence for the metabolic control of *gal* and *rfb* genes in EPS precursor synthesis and EPS production. The capsule of *S. pneumoniae* is important both for virulence and for asymptomatic colonization of the nasopharynx, and it is likely that capsule expression is reduced during colonization to facilitate adherence but enhanced during systemic infections to protect the bacterium from the host immune system. Regulation of capsule biosynthesis in *S. pneumoniae* was investigated by Robert Cartee (and Janet Yother, University of Alabama at Birmingham). Although 90 serologically distinct capsule types have been described for this organism, only two basic mechanisms of capsule biosynthesis are known. The most common mechanism involves the synthesis, transport, and polymerization of the capsule repeat unit and its transfer to the cell wall. In this case, four genes (*cpsA* through *cpsD*) involved in a tyrosine phosphorylation pathway regulate capsule biosynthesis, apparently through control of glycosyltransferase activity. Mutations in any of these genes modify the amount of capsule and/or its distribution on the cell surface and affect systemic virulence and colonization. The second pneumococcal mechanism of capsule synthesis is rare (serotypes 3 and 37) and involves a single glycosyltransferase for CPS biosynthesis and transport. The mechanism of type 3 capsule regulation is different from that for the majority of serotypes and involves substrate levels, in particular UDP-GlcUA, and a modulation of the turnover of the type 3 synthase.

GENETIC TOOLS AND APPLICATIONS: USING WHAT WE'VE LEARNED

From bacteriocins to transposons, genetic analyses of the streptococci have revealed the potential for useful applications in many areas. In particular, the lactic acid streptococci used in fermentation and bioprocessing, primarily *L. lactis* and *S. thermophilus*, have been the focus of considerable scientific investigation in recent years with the aim of developing genetic tools that can be exploited to reveal new industrial applications in agriculture and medicine (9, 15, 20).

An *L. lactis* strain isolated from Irish kefir grains by Paul Ross (Moorepark Research, Fermoy, Ireland) and Colin Hill (University College Cork, Cork, Ireland) produces a bacteriocin with a broad spectrum of activity against gram-positive bacteria. Like nisin, lacticin 3147 is a very effective bacteriocin that can be genetically transferred by conjugation. DNA sequencing of a conjugative plasmid, MRC01, present in this strain revealed a two-component peptide bacteriocin containing lanthionine residues. Recent efforts have analyzed the bacteriocin genetic determinants in detail to reveal two divergent promoters driving expression of two clusters, each containing five genes. Strains that overproduce lacticin 3147 have subsequently been generated. As a result of the genetic efforts to control gene transfer and expression of the bacteriocin, applications for the bacteriocin as a biopreservative in cheese, infant formula, natural yogurt, and pasteurized milk are rapidly developing. Veterinary applications were also highlighted, as lacticin 3147 has been successfully used in teat seals to inhibit mastitic streptococci and staphylococci.

One novel application for lactococci that is under development is use as a vaccine delivery vehicle and expression host for surface proteins. Jerry Wells (IFR, Norwich, United Kingdom) discussed the merits of lactococci, noting that these organisms are generally recognized as safe (GRAS), produce no endotoxins, and have substantial tools available for use as gram-positive expression hosts. Expanding on this utility, Wells reported on the use of lactococci as a vaccine discovery tool and the development of a LEEP screen (Lactococcal Expression of Exported Proteins) for identification of proteins that are exported by *S. pneumoniae* and are potentially involved in pathogenicity. Some of the proteins discovered in the LEEP screen have been shown to induce cross-protection against *S. pneumoniae* in mouse challenge studies. Exploitation of the nonpathogenic lactococci for expression of vaccines and surface proteins, both as vehicles for delivery and as genetic systems for discovery of new vaccines and cross-protective antigens, is a rapidly growing area.

For both dairy food fermentations and, more recently, bioprocessing applications (enzymes, vaccines, etc.), the practical use of *L. lactis* and *S. thermophilus* starter cultures continues to expand. These species, however, remain highly susceptible to bacteriophage attack, and efforts to find effective strategies to protect valuable strains continue. Sylvain Moineau (Université Laval, Québec, Canada) examined how *L. lactis* can escape phage attack during milk fermentation. The development of phage-resistant strains was initially based on the selection of spontaneous mutants; more recently, it has been based on the construction of engineered bacterial strains devoid of phage receptors. Natural, plasmid-encoded antiphage systems (adsorption inhibition, blocking of DNA injection, restriction-modification, and abortive infection) have also been used to construct resistant strains. The most effective system relies on abortive infection (Abi), and to date more than 20 distinct Abi systems have been isolated in *L. lactis.* However, phages evading the resistance mechanisms have emerged due to target alteration. New antiphage strategies based on genomic sequence analyses that have recently been investigated include titration of the phage origin of replication, inducible expression of a suicide system, and use of antisense RNA targeting essential phage genes. Joseph Sturino (and Todd Klaenhammer, North Carolina State University) reported on the effective use of antisense RNA to inhibit a major group of phages attacking *S. thermophilus*. Targeting the early phage DNA replication functions by antisense expression of primase and helicase genes resulted in significant levels of phage protection, reducing plaque-forming efficiencies 100- to 1,000-fold and preventing lysis in broth cultures. These early replication genes are highly conserved across *S. thermophilus* phages, and thus the strategy could have widespread utility in the protection of starter strains.

LOOKING TO THE FUTURE

At the conclusion of the meeting, it was clear that much of the future genetic research on the streptococci, enterococci, and lactococci will be genomics based. Understanding the distinctive commensal, pathogenic, and beneficial roles of these related bacteria represents one of the many challenges to microbiologists in this field. The increasing number of functional and bioinformatics tools available for investigating gene expression, regulation, and comparative genomics across these

genera are rapidly making the discovery of these answers a reality.

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