

MEETING REVIEW

The Seventh International Conference on the Genetics of Streptococci, Lactococci, and Enterococci[∇]

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The Seventh International Conference on the Genetics of Streptococci, Lactococci, and Enterococci, sponsored by the American Society for Microbiology, was held 18 to 21 June 2006, overlooking the sea in Saint Malo, Bretagne, France. The conference began in 1981 as the Streptococcal Genetics meeting, at a time when the genera *Enterococcus* and *Lactococcus* were still classified as *Streptococcus* spp. Through the years, this meeting has served as a quadrennial platform to showcase cutting-edge advances in the dissection of the underlying genetics of growth control, metabolism, extra-chromosomal elements, substrate utilization, competence development, antibiotic resistance, and virulence of this fascinating group of organisms. The penultimate meeting, held in 2002, focused heavily on then-recent advances made through bacterial and phage genome-sequencing projects. The meeting in Saint Malo highlighted the remarkable progress over the last 4 years in the areas of functional and comparative genomics that was spawned by the large-scale sequencing projects completed early this century. Some of these advances are summarized in this review of the oral sessions of the programs, and additional reports published in this issue of the *Journal of Bacteriology* provide a more detailed glimpse of the tremendous insights gained through genetic analysis of this unique group of eubacteria.

KEYNOTE SESSION

In 1944, the team of Avery, MacLeod, and McCarty reported on the chemical identification of the substance that induces the transformation of *Streptococcus pneumoniae* (3). Their identification of DNA as the hereditary material not only launched the field of streptococcal genetics, but also provided a foundation for many of the seminal discoveries in biology and medicine of the past 62 years. In 2005, the scientific community lost a member of that team, Maclyn McCarty. Mac brought both his biochemical expertise and personal drive to bear on understanding the substance derived from killed bacteria that led to the fatal bacteremia by otherwise nonvirulent bacteria

injected into Griffith's mice. Mac spent most of his career as a physician-scientist at The Rockefeller University and was a regular attendee of the ASM Streptococcal Genetics meetings. In the opening presentation in Saint-Malo, Mac's longtime colleague and friend, Vince Fischetti, presented a sketch of Mac's lifetime of contributions to the understanding of streptococci and the diseases they cause in humans (26). Rheumatic fever, the often devastating illness that can follow a *Streptococcus pyogenes* infection, was one challenging puzzle to which Mac brought important new insights through study of the patient's immune response and the biochemistry of the bacterium's structural features.

The opening session also included talks on comparative genomics and population genetics. Kira Makarova (working with Eugene Koonin, National Center for Biotechnology Information) provided highlights of a comparative genomic analysis of nine species of lactic acid bacteria (LAB). This work represents one of the most extensive comparative analyses of a group of closely related prokaryotic genomes. A central theme in the evolution of LAB is the loss of ancestral genes and metabolic simplification as a consequence of cellular adaptation to nutritionally rich environments. Past adaptation to nutritionally rich, microaerophilic environments was also evident in the duplication and acquisition of genes related to sugar utilization, nitrogen assimilation, and other functions. The presence of genes encoding putative bacteriocins in most strains probably reflects long-term adaptation of these cells to growth and competition in complex microbial communities. *Lactobacillales*-specific clusters of orthologous genes (LaCOGs) from proteins encoded by the sequenced genomes were defined (67). Only 35% of LaCOGs corresponded to the general COG data set, 22% had no counterpart in the general COG set, and 11% appeared to be specific to the *Lactobacillales* species cluster. Thus, the LaCOGs will be a powerful resource for genome annotation and evolutionary analysis of the *Lactobacillales* (47).

The past several years have witnessed the explosive growth of sequence-based typing schemes for precise identification of bacterial isolates of a known species. Perhaps foremost among these tools is multilocus sequence typing (MLST), typically based on seven neutral housekeeping genes (1). That MLST can be a lot more than just a tool for cataloguing strains was made very clear by Brian Spratt (Imperial College, London,

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United Kingdom). Sequence data generated by MLST permits evaluation of the primary mechanism underlying genetic change in a bacterial population, namely, recombination versus mutation (22). MLST can also be used to predict the founders of medically important clones and their patterns of evolutionary descent. This was illustrated for the ST81 clone of *S. pneumoniae*, which has acquired multiple drug resistance genes; has appeared with several different capsular polysaccharide types; has undergone changes in housekeeping alleles, mostly by recombination; and has traveled the globe in the ~30 years following an unremarkable existence in Spain as serotype 23F-1. Comparison of MLST data from *Streptococcus pneumoniae*, *Streptococcus suis*, *S. pyogenes*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Enterococcus faecalis* demonstrated that there is wide variance among species in the relative impact of recombination on their evolution. Problems inherent in the “species concept” (25) were illustrated by the intermingling of housekeeping gene trees for *Streptococcus mitis*, *Streptococcus oralis*, and *S. pneumoniae*. MLST has also helped to resolve the taxonomic confusion surrounding the “atypical” pneumococci, which are now recognized as *Streptococcus pseudopneumoniae*. Bacteria are neither strictly asexual (i.e., clonal) or fully sexual (e.g., diploid), but rather undergo genetic diversification, in part by localized recombination. Rooted in the basic principles of the Fisher-Wright model for sexual organisms, the gap in population genetic theory for bacteria is beginning to be filled in with a new multilocus infinite alleles model of bacterial evolution (24). Recent advances in mathematical modeling have also led to a simulation of the evolution of a bacterial population over time, providing a visual display of how sequence clusters that drift apart are pulled back together by the cohesive force of recombination.

POPULATION BIOLOGY AND EVOLUTION

A bacteriophage attack on LAB can spoil a fine French cheese well before it has ripened and aged via fermentation. The economic pressures faced by the dairy industry have helped to advance phage research applicable not only to *Lactococcus lactis* and *Streptococcus thermophilus*, but also to human pathogens, such as *S. pyogenes* and *Streptococcus mutans*. As outlined by Louis-Charles Fortier (University Laval), there are a large number of genetically diverse groups of lactococcal phage (21). Comparative genomics studies have uncovered several interesting parallels between *L. lactis* and *S. pyogenes* phage and between *S. thermophilus* and *S. mutans* phage. Bacteriophage control has important implications, not only for the dairy industry, but also for novel therapies against bacterial infections of mammalian hosts (23).

Genetic steps that allow a bacterium to exploit a new ecological niche were delineated in the presentation by Rob Willem (University Medical Center Utrecht). The highly successful clonal complex (CC17) of hospital-adapted *Enterococcus faecium* has become the vancomycin-susceptible precursor for many vancomycin-resistant enterococci. Interestingly, the ecological abundance of CC17 is much higher in the hospital than in farm animals, where the use of glycopeptide antibiotics in feed has been controversial. Hospital adaptation by CC17—a highly specialized enterococcal genetic subpopulation—appears to be a multistep process in which genes acquired by

horizontal transfer provide a selective advantage in exploiting the hospital environment. This leads to an increase in clonal frequency that facilitates further adaptive possibilities (72). Genes encoding resistance to other antibiotics (ampicillin and ciprofloxacin) and virulence factors (the *esp*-associated pathogenicity island) were acquired by CC17 organisms in their adaptation to the hospital. Comparative genomic hybridization using microarrays has identified ~100 candidate genes, some of which may have roles in increasing the fitness of CC17 in the hospital environment. Through better understanding the CC17 precursors of vancomycin-resistant enterococci, it may be possible to devise strategies that minimize the emergence of untreatable nosocomial infections.

The pneumococcal conjugate vaccine (PCV7) consists of seven capsular polysaccharides covalently linked to diphtheria toxoid. A few years prior to its licensing in 2000, a mathematical model predicted that organisms of the serotypes included in PCV7 would likely be replaced by organisms of other serotypes (41). Bernie Beall (Centers for Disease Control and Prevention) reported that PCV7 has already been a huge success in its target age group (children) and has also led to a reduction in adult disease via a herd effect. However, serotype 19A, a capsular type not included in PCV7, has recently emerged as the most prevalent serotype associated with pneumococcal invasive disease in the United States. This new virulent clone appears to have arisen following horizontal transfer of a 39-kb DNA fragment containing serotype 19A capsule and PBP1a genes from an ST199 donor strain to a serotype 4-ST695 recipient strain. Serotype 4 capsular polysaccharide corresponds to one of the seven vaccine serotypes successfully targeted by PCV7. This single genetic replacement event appears to account for the emergence of this highly virulent vaccine escape mutant on the ST695 background.

As the number of complete genome sequences increases, comparative genomic analysis becomes more powerful. Matthew Holden (The Wellcome Trust Sanger Institute, Cambridge, MA) compared the genome sequences of representative isolates of four streptococcal pathogen species (*S. pyogenes*, *S. uberis*, *S. suis*, and *Streptococcus equi*) that primarily infect four different host species (human, bovine, porcine, and equine, respectively). Species comparisons of orthologues revealed core and accessory genes that are species specific, thereby providing new insights into specific niche adaptations and pathogenic processes. Mobile genetic elements contribute to the accessory gene makeup. The proportion of the genome comprised of mobile genetic elements was found to be higher for *S. pyogenes* and *S. equi* than for *S. suis* and *S. uberis*.

GENE TRANSFER, COMPETENCE, AND RECOMBINATION

Don Morrison (University of Illinois at Chicago, Chicago, IL) provided an overview of the biology of competence in the pneumococcus. This transient phenomenon develops suddenly as a consequence of the accumulation of a peptide pheromone, CSP, which participates in cell-cell signaling through a positive-feedback loop and a transcriptional cascade involving a two-component signal transduction system (ComDE) and an alternative sigma factor (ComX). Transcription of ~100 genes in >30 operons is briefly induced (16, 53). While the wide-

spread display of competence in this species implies a strong selective pressure for its maintenance, the observation that ~70% of the induced genes are not required for DNA uptake and processing suggests that competence does not develop only for the purpose of genetic transformation. Recent reports revealing that competence is also associated with a transient propensity to cause lysis of noncompetent neighbors (referred to as allolysis or fratricide) are consistent with this view. Six of the CSP-induced genes are now known to participate either in allolysis (*lytA* and *cbpD*, which encode amidases, and *cibA-cibB*, which encode a two-peptide bacteriocin) or in establishing immunity against lytic factors in competent cells (*comM* and *cibC*) (28, 30, 33). This remarkable form of cooperative aggression might serve to sample the genomes of nearby bacteria. However, allolysis of a subpopulation may also contribute to interactions with the host in ways unrelated to gene transfer. It could provide, for example, a mechanism for the regulated release of the intracellular virulence factor pneumolysin. At least 13 of the operons induced by CPS are devoid of genes implicated in the regulation of competence, DNA uptake, genetic recombination, lytic interactions, or immunity. Additional unknown but similarly coordinated activities may remain to be discovered. Some could account for the recent observation that administration of CSP can grossly alter the outcome of experimental sepsis.

Coregulation of bacteriocin production and competence is also known to occur in *S. mutans*. Fengxia Qi (University of California at Los Angeles) reported recent investigations of the underlying regulatory mechanism. Bacteriocin (mutacin IV) production and competence development were previously shown to be coordinated through transcription regulation by a three-component quorum-sensing system called *comCDE*. The *comC*-encoded CSP has now been found to regulate a group of bacteriocin-like genes, in addition to mutacin IV production. Interestingly, the bacteriocin-encoding genes respond immediately to CSP, whereas the response of the *comC* gene is delayed for 1.5 to 2 h after CSP addition (37). Moreover, expression of the bacteriocin genes absolutely requires the presence of *comE*, while deletion of *comE* enhanced *comC* gene expression to the same level as addition of CSP. Point mutations mimicking the phosphorylated and nonphosphorylated forms of ComE were used to demonstrate that transcriptional activation of the bacteriocin genes requires the CSP-induced ComDE phosphorylation cascade (and binding of ComE~P to direct repeats identified in front of the CSP-induced genes) and that the same system induces competence gene expression indirectly through relief of ComE-dependent repression of *comC*. These findings, which are in contrast to the *S. pneumoniae* paradigm, are fully consistent with recent observations showing that *S. mutans* ComDE is not the orthologue of *S. pneumoniae* ComDE but of BlpRH (42), a two-component system controlling expression of bacteriocin-like genes. The regulatory link between *S. mutans* ComCDE and the competence-specific sigma factor ComX remains to be understood.

Conjugative transposons are believed to play important roles in the shaping and the evolution of variable genomic islands in bacterial chromosomes. Gérard Guédon (Université Henri Poincaré, Nancy, France) provided support for the view that several genomic islands of *S. thermophilus* are integrative and conjugative elements (ICEs) or ICE-derived elements (52)

evolved by deletion and tandem accretion resulting from site-specific recombination. Excision of ICEs involves tyrosine-integrase-mediated recombination between two 27-bp direct repeats included in the two attachment sites of ICEs, *attL* and *attR*. *cis*-mobilizable elements would presumably arise from ICEs by deletion of the conjugation and recombination modules, whereas integration of an ICE acquired by conjugation by site-specific recombination between *attI* of its circular form and an *att* site flanking a resident *cis*-mobilizable element or ICE would lead to evolution of a preexisting genomic island.

Philippe Glaser (Institut Pasteur, Paris, France) described the characterization of a novel class of conjugative transposons identified in highly variable islands of the *S. agalactiae* genome, as well as in group B streptococcus (GBS) strains and in *Streptococcus gallolyticus*. These transposons harbor 23-bp inverted repeats at their extremities and generate 9-bp duplications upon integration. In contrast to all gram-positive conjugative transposons described so far, which require the action of site-specific serine or tyrosine recombinases, each of the putative transposons potentially encodes a transposase containing the DDE motif. PCR-based detection of the circular forms of these elements suggested that the transposases are active. In addition, one of these putative transposons (Tngbs2) was shown to be transmissible among *S. agalactiae* strains by filter mating, consistent with the presence of open reading frames encoding proteins similar to the conjugation proteins TraG and TraE of the *E. faecalis* conjugative plasmid pCF10. Altogether, these observations support the view that these elements constitute a new class of conjugative transposons involved in the evolution of group B streptococci.

Sergey Artiushin (University of Kentucky) reported an analysis undertaken to understand the features unique to *S. equi* that account for its enhanced virulence and narrow adaptation to equids compared with *Streptococcus zooepidemicus*. The latter is an opportunistic pathogen of the respiratory and reproductive tracts that lacks host specificity and is antigenically highly variable. The use of subtractive libraries made by using *S. equi* as a tester and *S. zooepidemicus* as a driver DNA led to the identification of mobile-element sequences accumulated in the genome of *S. equi*. Inactivations of a number of potentially important genes through mobile-element expansion and phage acquisition were proposed to represent important events in the formation of the clonal, highly host-adapted *S. equi* from an ancestral *S. zooepidemicus*.

The resolution of circular chromosome dimers accidentally produced in bacteria is essential for correct chromosome transmission to daughter cells. In the paradigmatic *Escherichia coli*, chromosome dimer resolution is achieved through site-specific recombination at the *dif* site, a 28-bp sequence located near the terminus of chromosome replication. Two tyrosine recombinases, XerC and XerD, catalyze a concerted strand exchange reaction. This reaction is intimately coupled with cell division by the septal DNA translocase FtsK, which brings together the two *dif* sites of a dimer at the division septum and activates cleavage through direct interaction with XerD. Pascal Le Bourgeois (and Paul Ritzenthaler; LMGM CNRS-Université Paul Sabatier, Toulouse, France) reported that in contrast to this paradigm, streptococci and lactococci, but not enterococci, use an exotic 31-bp recombination site, *dif*_{SL}, and only one tyrosine recombinase, XerS, encoded by a gene adjacent to *dif*_{SL}.

SENSING AND SIGNALING

Two-component signal transduction systems are likely to play a key role in the adaptation to different environments within the human host by many streptococcal species that are otherwise devoid of stationary-phase or stress-responsive secondary sigma factors. June R. Scott (Emory University) described recent findings concerning the role of the CovRS system of *S. pyogenes*. This regulation system has been shown by microarray analysis to regulate ~15% of the M1 group A streptococcus (GAS) genome, including genes encoding several virulence factors. Unlike most response regulators that activate gene expression, CovR functions chiefly as a repressor of transcription. Inactivation of CovR by its cognate sensor kinase, CovS, is required for the growth of GAS under stress conditions (18). Inactivation of *covS* prevents growth under mild stress conditions (pH 6, 40°C, or high salt), suggesting that CovR controls genes important for stress responses. One such operon, *rscAB*, encodes a homologue of the ABC exporter MDR1 (17). It is speculated that this operon is required for the export of products that are toxic under stress conditions or that it affects the movement of specific membrane lipids, thus allowing appropriate membrane composition and fluidity for growth at high temperatures.

In contrast to most quorum-sensing systems that involve a single cell type whose population density in a particular niche is sensed by using a signal molecule, the pheromone system regulating conjugative transfer of the antibiotic resistance plasmid pCF10 in *E. faecalis* involves two distinct populations, donor cells and plasmid-free recipients. Gary Dunny (University of Minnesota) reviewed the most recent data on the molecular mechanisms regulating the induction of a pCF10-containing cell in response to the mating pheromone cCF10 produced by plasmid-free recipient cells. This induction is controlled by a complex sensing system involving multiple sets of positively and negatively acting regulatory molecules. They include two plasmid-encoded factors (iCF10 and PrgY) and active and inactive forms of the cytoplasmic pheromone receptor PrgX. Recent structural analysis of complexes between PrgX and cCF10 or iCF10 have revealed that both peptides interact with the same peptide-binding pocket of PrgX but induce different conformational alterations in the protein, resulting in opposite effects on its oligomerization state (61). Another key parameter, the balance of iCF10 and cCF10 in the growth medium, depends on the level of expression of the *prgQ* locus encoding iCF10 production, as well as on the control of endogenous pheromone production by PrgY. In addition, human plasma induces pCF10 transfer and expression of the aggregation substance Asc10 by causing endogenous pheromone induction, probably by disrupting the iCF10/cCF10 ratio (14). Altogether, the data indicate that this complex control system serves multiple functions in that it allows the cell to detect the presence of recipient cells in close proximity and to ascertain that its growth environment is the mammalian bloodstream. The pheromone-inducible aggregation substance Asc10 increases virulence in several animal models, consistent with the view that the sensing system has evolved to control not only conjugation functions, but also virulence.

The production of bacteriocins by LAB is of great interest because of their potential use as natural preservatives in food

products. Laetitia Fontaine (and Pascal Hols; Université Catholique de Louvain, Louvain, Belgium) reported the study of a class II bacteriocin locus identified in the genome of *S. thermophilus* (9) on the basis of homology to the previously characterized *blp* locus of *S. pneumoniae* (20). These loci contain genes encoding a typical quorum-sensing regulatory module composed of a conserved two-component system, BlpRH; the corresponding inducer pheromone, BlpC; and its dedicated proteolytic ABC transporter, BlpAB. A variable number of genes encoding bacteriocin-like peptides containing a typical double-Gly leader are located downstream of *blpRH* in three *S. thermophilus* strains (31). The addition of synthetic BlpC to one of these strains was shown to induce transcription of the bacteriocin-encoding genes and the production of bacteriocins active against *S. thermophilus*, as well as other species. Modulation of the inhibitory spectrum of *S. thermophilus* could be demonstrated through the combination of different *blp*-encoded bacteriocins.

Defining the regulon under the control of a two-component regulatory system is not an easy task. It proved to be even more complicated in the case of CiaRH of *S. pneumoniae*, which has been implicated in β -lactam resistance, protection from a variety of lysis-inducing conditions, competence, and virulence. Transcriptome analyses (16, 43, 59) and genomewide solid-phase DNA binding assays with the response regulator CiaR (43) resulted in inconsistencies in the definition of the CiaR regulon and revealed no obvious link to most of the observed phenotypes. Reinhold Brückner (and Regine Hakenbeck; University of Kaiserslautern, Kaiserslautern, Germany) presented new data that may open the way to a better understanding of CiaR-mediated regulation. Briefly, a CiaR-binding sequence was first identified by gel shift analysis and mutagenesis. The genome sequence of *S. pneumoniae* R6 was then searched using this sequence, leading to the detection of several new putative CiaR-regulated promoters. Intriguingly, the five strongest CiaR-regulated promoters were found to be located upstream of genes that were apparently not regulated by CiaRH but expressed from CiaR-independent promoters located closer to the respective genes than the CiaR-controlled promoters. Inspection of the DNA sequences immediately downstream of these CiaR-dependent promoters revealed the capacity to code for ~100-nucleotide-long RNAs with a high degree of similarity to each other. Characterization of these RNAs will undoubtedly clarify the regulatory link to CiaRH-dependent phenotypes.

The regulation of nitrogen metabolism is of paramount importance for the survival of bacteria in a variety of different environments. Oscar Kuipers (University of Groningen, Groningen, The Netherlands) described the results of detailed comparative studies of the regulatory roles of GlnR and CodY in *L. lactis* and *S. pneumoniae*, combining the use of microarrays, *lacZ*-reporter fusions, in vitro analysis of protein-DNA interactions, and mouse infection. A conserved CodY box identified in *L. lactis* could also be found in relevant promoters in *S. pneumoniae* (19). A similarly identified GlnR box was also found to be conserved in both species (36, 38). Dual regulation by both CodY and GlnR of a few genes differing between *L. lactis* and *S. pneumoniae* pinpointed presumably important control points of the cellular nitrogen status of each species. Mouse infection studies and in vitro assays of adhesion to

human nasopharyngeal cells provided evidence that both CodY and GlnR regulons have a significant impact on the virulence of *S. pneumoniae*.

CELL SURFACE BIOGENESIS AND FUNCTION

The fourth oral session at the conference was dedicated to current advances in the understanding of protein secretion and the biogenesis and functions of cell surface proteins of pathogenic streptococci. Jeannine Brady from the University of Florida expanded on her recently published work (29) that detailed fundamental differences in the requirements for the protein secretion machinery of *Streptococcus mutans*. Specifically, deletion of critical components of the signal recognition particle cotranslational protein secretion pathway (Ffh and FtsY) that were believed to be required for viability of all forms of life revealed that these components are dispensable in *S. mutans*. The molecular basis for this was disclosed by demonstrating that *S. mutans* carries two copies of genes encoding homologues of the YidC/OxaA/Alb3 family of membrane proteins mediating protein translocation in prokaryotes, mitochondria, and chloroplasts, respectively. Inactivation of *yidC2* and *ffh* in a single strain was not possible, demonstrating that YidC2 can compensate for the lack of an intact signal recognition particle, and vice versa. It was also notable that the duplication of *yidC* genes occurs in the genomes of related streptococci for which complete sequence information is available. Thus, fundamental differences are evident in the ways in which this unique group of organisms moves proteins to the cell surface and beyond.

“Streptococcologists” have led the way in the discovery and dissection of surface-localized, glycosylated proteins in bacteria. Paul Sullam (University of California at San Francisco) provided a detailed update on progress on the interrelationship of secretion and glycosylation of the GspB surface glycoprotein of *Streptococcus gordonii*. GspB is encoded in a locus containing genes required for transport and glycosylation and has served as the paradigm for proteins in related streptococci and staphylococci (7). GspB is secreted through an accessory Sec system encoded in the *gspB* gene cluster, and glycosylation is critical for the stability of the adhesin (5, 65, 66). Still, GspB can also exit the cell through the canonical Sec system. However, glycosylation of GspB prevented efficient export through the canonical system, implying that the accessory Sec system has evolved specifically for export of glycosylated proteins in a posttranslational secretion pathway. Experimental evidence was presented to show that GspB appears to be targeted to this specialized accessory secretion pathway by an atypical 90-amino-acid signal sequence (8).

Hui Wu (University of Alabama at Birmingham) continued the excursion through the genetics and function of glycosylated streptococcal surface proteins and of accessory proteins involved in their modification and transport. The Fap1 adhesin of *Streptococcus parasanguis*, which appears to have been acquired by horizontal gene transfer (64), is a 2,570-amino-acid fimbria-associated protein that is heavily decorated with carbohydrate through O linkages. Two genes, *gtf* and *secY2*, the latter encoding an accessory Sec system similar to that described above, are differentially involved in Fap1 glycosylation (15). Loss of SecY2 led to elimination of one type of glycosyl

moiety from the mature Fap1 protein, but the partially glycosylated precursor was still exported to the cell surface. In contrast, mutants lacking the *gtf* gene produced a nonglycosylated Fap1 precursor. Notably, biofilms formed by the *gtf* and *secY2* mutants exhibited markedly different architectures, clearly demonstrating the importance of posttranslational modification and proper secretion and localization of Fap1 for the pathogenic potential of *S. parasanguis*.

Caparon and coworkers first described what is an apparent specialized protein translocation apparatus located in foci in *Streptococcus pyogenes*, known as the ExPortal (57). Among the proteins identified as colocalizing at the ExPortal is sortase, an enzyme that catalyzes the covalent linkage of cell surface proteins to peptidoglycan through the threonine residue in conserved LPXTG sequences found in a subset of secreted proteins of streptococci and related organisms (48). Kimberly Kline from Washington University in Saint Louis, MO, described attenuation of virulence in a mouse urinary tract infection model resulting from inactivation of sortase of *Enterococcus faecalis*. To determine whether sortase could be localized to the ExPortal, immunolocalization studies were performed to demonstrate that sortase and SecA colocalized at a punctate site on the cells characteristic of the ExPortal. It was also noted that proteins that are known to localize at the ExPortal carry an unusually large number of positively charged residues in juxtaposition to a C-terminal transmembrane domain. Mutation of these residues in sortase affected ExPortal localization, and a significant number of proteins carrying this signature, termed the ExPortal targeting sequence, could be identified in the genome. Preliminary information showing that there was a preponderance of anionic lipid localized at the ExPortal suggested that perhaps the positively charged ExPortal targeting sequence facilitates the interaction with the export site, consistent with a recent report on localized protein secretion in group A streptococci (13).

The final presentation of this oral session was given by Katrin Dinkla from the German Research Center for Biotechnology and highlighted critical structure-function relationships in the fibronectin binding proteins of group A and G streptococci (35, 69). *S. pyogenes* produces streptococcal fibronectin binding protein I (SfbI), which triggers adherence and the invasion of epithelial cells (45). An apparent homologue of SfbI, GfbA, was identified in group G streptococci and was shown to enhance adherence to skin fibroblasts. A knockout mutant lacking GfbA and heterologous expression of *gfbA* in the noninvasive host *Streptococcus gordonii* supported the notion that GfbA contributes significantly to the ability of cells to invade human epithelial cells. Importantly, *S. gordonii* expressing *sfbI* was targeted to caveosomes, whereas GfbA-producing recombinant *S. gordonii* localized in lysosomes. GfbA and SfbI are 97 to 100% identical in the fibronectin binding domains and the proline repeat regions but differ significantly (60% homology) in the aromatic-rich domains, suggesting that the differences in subcellular targeting observed between the parental organisms and recombinant *S. gordonii* strains may be attributable to differences in the aromatic domains of these two surface proteins.

PATHOGENESIS

Serine/threonine kinases, which are widely used in the eukaryotic world to modulate gene expression, have recently been detected in a variety of prokaryotes. A single serine/threonine kinase, Stk1, was identified by Lakshmi Rajagopal and coinvestigators in Craig Rubens' laboratory at the University of Washington in Seattle (54). An apparent serine/threonine phosphatase (Stp1) was also identified in the same strain. Stk1 mutants showed a substantial decrease in virulence and survival in blood and displayed enhanced susceptibility to phagocytosis. Notably, Stk1 regulates the expression of *cylE*, encoding the β -hemolysin of the organism, but this regulation appears to be exerted through CovR of the CovRS two-component system. CovR has been shown to regulate 138 genes in GBS, so a role for Stk1 in global regulation of virulence appears likely.

Genomics and microarrays have revolutionized the study of pathogenic streptococci and have allowed the identification, for example, in the case of Stk1 in GBS, of central regulatory elements globally regulating virulence gene expression. Paul Sumby from Jim Musser's group in the Methodist Hospital in Houston, TX, detailed their work on ex vivo and in vivo models for transcriptome profiling of GAS. Growth of GAS in blood or saliva resulted in major remodeling of the transcriptome of the organisms. Over 500 genes were differentially expressed in blood, including the streptolysin S operon and pilus proteins, with an overall remodeling to adapt to this new microenvironment. Growth in saliva also resulted in dramatic changes in gene expression (60), including a 2,300-fold increase in *speB*, encoding a cysteine proteinase. Longitudinal analysis of the transcriptome in primates revealed distinct responses of the organisms to environments encountered during colonization, during the acute phases of the disease, and beyond (70). Perhaps not surprisingly, two-component systems appear to be heavily involved in these responses in vivo, and evidence was also presented that prophage may be induced in vivo during infection.

Tim Mitchell of the University of Glasgow gave an excellent overview of the pathogenesis of pneumococcal diseases. A major focus of the presentation was to highlight the tremendous genetic heterogeneity and plasticity of pneumococci. For example, PspC of the pneumococcus is a surface protein that contributes to complement and phagocytosis resistance through binding of factor H and the C3 component of complement. In addition, this protein contributes to adherence and invasion through binding of immunoglobulin receptor (34). However, inactivation of *pspC* in four different strains of pneumococci had dramatically different effects on pathogenesis. Interestingly, a population-based study using comparative genome hybridization and multilocus sequence typing showed that there are three new regions of high genetic diversity in identical MLST types and capsular serotypes. Importantly, these strains behave dramatically differently in animal models.

Building on the concept of bacterial adaptation to host microenvironments, Claire Poyart presented highly significant findings about oxygen, aerobic metabolism, and virulence of GBS. Notably, GBS, which like other streptococci are generally considered to rely exclusively on fermentation for ATP generation, were shown to be able to assimilate heme and

menaquinone from blood to activate a respiratory chain, doubling the growth yield compared with fermentation alone (73). Mutations in *cydA*, encoding a subunit of cytochrome *bd* quinol oxidase, resulted in exclusively fermentative growth but also attenuated virulence in a neonatal-rat sepsis model. Attention then turned to the H₂O-forming NADH oxidase Nox-2. When the *nox2* gene was inactivated, growth of the organisms in elevated oxygen concentrations was affected and the organisms acquired sensitivity to paraquat and peroxide. Interestingly, glucose utilization and lactate production were not affected in the *nox2* mutant strain, but acetate was not produced. Also, good growth in the presence of oxygen by the mutant could be restored by the addition of exogenous acetate, which was shown to be, at least in part, attributable to provision of adequate levels of acetyl-coenzyme A to support fatty acid biosynthesis. Addition of menaquinone and heme could also bypass the *nox2* defect, and *nox2* mutant strains showed decreased virulence and lower numbers of organisms in the blood and brains of infected animals.

Jennifer Mitchell presented the final talk in this session, detailing a novel method for biogenesis of platelet binding proteins on the surface of *Streptococcus mitis*, an oral streptococcus that is a significant contributor to bacterial endocarditis. Two surface proteins, PblA and PblB, are required for platelet binding and are encoded on the lysogenic bacteriophage SM1 (6). Neither of the proteins is translated with a classical signal sequence, nor do they contain LPXTG anchor sequences. Induction of the phage by mitomycin C or UV light enhanced the levels of surface-localized PblAB. Inactivation of the holin-lysin genes of phage SM11 demonstrated that these proteins were required for release of PblAB from the cells and decreased platelet binding activity. Pure PblA and PblB added to mutant cultures became associated with the surface of *S. mitis*, possibly through GW-rich (glycine-tryptophan) sequences implicated in choline binding. Thus, populations of *S. mitis* can enhance their platelet binding potential by prophage induction to release PblA and PblB, a form of "bacterial altruism" that may be used in vivo to modify the adhesive repertoire of the organisms under conditions that trigger phage induction. The presence of these virulence genes on a phage could allow dissemination to related organisms.

PATHOGENESIS AND GENETICS

S. pyogenes is a highly prevalent pathogen that usually causes only a mild disease in its human host. However, there are an unlucky few who develop toxic shock syndrome. According to Malak Kotb (University of Tennessee at Memphis), the reasons why this is so are multifactorial and depend on the infecting strain, the genetic background of the host, and the unique host-pathogen interaction that ensues. The M1T1 strain has been predominant in toxic shock syndrome cases for more than 25 years, and its associated phage are highly mosaic in structure, harboring an array of toxin genes (4). Several of the toxin genes encode superantigens that interact with HLA class II antigens, whereby different HLA types can confer risk or protection from severe invasive streptococcal disease. A mouse sepsis model was used to identify susceptible and resistant mice generated by F₂ crosses over many generations, leading to quantitative trait loci that can be mapped. Invasive-disease

severity is also influenced by the amount of SpeB cysteine protease that is produced by the infecting strain, and high levels of SpeB production are associated with less severe disease because other streptococcal virulence factors are degraded.

The structure and function of the pathogenicity island (PAI) of *E. faecalis* were detailed by Janet Manson (Schepens Eye Research Institute). This 153-kb PAI has many of the expected features, such as altered G+C content and ends having homologies to other mobile genetic elements. It also has genes for some well-characterized virulence determinants, including aggregation substance, cytolysin, and Esp, which enhances colonization in the urinary tract. A bile salt hydrolyase gene was examined as a novel virulence trait. The bile salt hydrolase was found to reduce bile toxicity by cleavage of a side group that otherwise enhances bile salt solubility in the low-pH environment of the gastrointestinal tract. Stress response genes also appear to be components of this PAI.

Why does *S. pyogenes* grow in chains? This is a seemingly simple question with an elusive answer. One possibility lies in the sacrifices that are made when phage lysogenize, producing host toxins that enhance the survival of the remaining intact bacterial cells. In searching for proteins secreted by *S. pyogenes* during contact with a pharyngeal cell line, the laboratory of Vince Fischetti (The Rockefeller University) uncovered two phage products, a toxin (SpeC) and a DNase (Spd1) (11). Unlike many other toxinogenic phage, the induction of lysogenic phage in streptococcal-epithelial cell coculture demonstrates the lack of a requirement for damage to the bacterial chromosomal DNA. The phage-inducing factor is diffusible but has not yet been identified. New phage infection was shown to occur with high efficiency using marked donor and recipient bacterial strains following their colonization of the oropharyngeal cavity of the mouse. Perhaps the role of DNase is to free the phage particle after bacterial-cell lysis. For the *S. pyogenes* bacteriophage, the mammalian host appears to be an essential component of its life cycle.

ADAPTATION AND LIFESTYLE

Like many eubacteria, streptococci and related microorganisms display remarkable adaptive flexibility to different environmental challenges or lifestyle changes. Since each of these cellular functions is scripted by the dynamic and coordinated expression of various gene regulons, detailed knowledge of gene content and global gene regulation in streptococci, lactococci, and enterococci continues to generate astounding new insights into the biology of these important microbes. Pierre Renault (INRA, Jouy en Josas, France) described key similarities and differences between lactococcal and *S. mutans* regulons for nitrogen assimilation and amino acid biosynthesis. The pleiotropic transcriptional repressor CodY, for example, is highly conserved in many low-G+C gram-positive species (55). Transcriptome analysis of *L. lactis* has shown that CodY is involved in global control of nitrogen metabolism and represses over 100 genes in response to the intracellular accumulation of branched-chain amino acids, particularly isoleucine (27). Pathways subject to CodY regulation in lactococci included those associated with protein degradation, peptide and amino acid uptake, and de novo amino acid biosynthesis.

Genome scans have identified the CodY-binding site, first defined in *L. lactis* via a combination of bioinformatics and genetics experiments, in the promoter region of nitrogen-related genes in streptococci and other gram-positive bacteria. However, putative CodY targets in lactococci and streptococci also show interesting differences that suggest this repressor may control other cellular processes, such as cell communication or host interaction, in one genus but not the other. Renault described additional differences between lactococci and *S. mutans* in metabolic regulation of sulfur-containing amino acids. Control of methionine metabolism in gram-positive bacteria, for example, is typically effected by S-box or T-box mechanisms, whose actions are exerted at the level of premature transcriptional termination. However, Rodionov et al. (56) found that streptococci, lactococci, and enterococci lacked S boxes, as well as methionine-specific T boxes, but noted that streptococci did contain a palindromic motif, termed the MET box, in the upstream sequence for most Met-related genes. Only one copy of the MET box was located in *L. lactis*, however, and Renault and coworkers determined that lactococci utilize a single transcriptional activator, FhuR, to regulate most genes involved in sulfur amino acid biosynthesis and that the FhuR consensus binding site is different from the MET box (62).

An even more pronounced paradigm difference was described by Jose Lemos (and Robert Burne; University of Florida), who reported on the mechanism by which *S. mutans* and related species control intracellular levels of (p)ppGpp alarmone. This alarmone acts as a global regulator for microbial adaptation to numerous adverse environmental conditions, including some that result from biofilms or host colonization. In gram-negative cells, synthesis and hydrolysis of (p)ppGpp is catalyzed by two proteins, RelA and SpoT. RelA acts as a synthetase, while SpoT is a bifunctional enzyme with synthetase and hydrolase activities (32). In streptococci and other gram-positive bacteria, however, (p)ppGpp synthetase and hydrolase activities are both provided by a bifunctional RelA enzyme (44). Curiously, *S. mutans relA* null mutants were found to still be able to produce (p)ppGpp but were defective in biofilm formation (40). Here, Lemos described genes for two novel *relA*-like proteins (termed RelP and RelQ) that were identified in the *S. mutans* genome. One of these genes, *relP*, is under the control of a cotranscribed two-component regulatory system. Mutation analysis showed that inactivation of *relP* and its cognate two-component regulatory system abolished (p)ppGpp production in *S. mutans*, while *relQ* inactivation produced cells whose phenotype was similar to that of the *relA* mutant. Protein homology searches identified RelP and RelQ paralogs in other low-G+C species of bacteria, suggesting more widespread deviation from the current dogma that RelA is the only enzyme for (p)ppGpp synthesis in bacteria.

Stress adaptation and other cellular processes associated with the transition by streptococci between planktonic and more environmentally resistant sessile (biofilm) lifestyles continues to attract considerable research interest. The structurally complex nature of biofilms provides greater protection against environmental extremes and antimicrobial agents and makes them difficult to control as sources of contamination or disease. The transition to sessile life is an organized process that starts with a cell density-dependent process called quorum

sensing (2), and one of the best-understood mechanisms for quorum sensing in streptococci involves the induction of genetic competence. Marco Oggioni (University of Siena, Siena, Italy) and coworkers analyzed *S. pneumoniae* transcription during blood and tissue infections in mice and found that the gene expression profile for cells in blood was very similar to that of laboratory-grown planktonic cells. In contrast, cells recovered from tissue infections showed an expression profile that was more akin to that seen in a biofilm model. Interestingly, biofilm induction in the model required the quorum-sensing competence-stimulating peptide CSP, and CSP also increased cell virulence in the pneumonia tissue infection model but reduced virulence in the sepsis model. Challenge studies with a *comD* mutant (deficient in CSP signaling) showed the opposite response in tissue and sepsis models, and these observations were further confirmed in experiments using laboratory-grown cells from biofilm or planktonic culture (51). Based on these findings, Oggioni postulated that virulence in pneumococci is directly related to lifestyle, where sepsis infections are favored by a planktonic state and tissue infections by a biofilm-like state.

The implication of CSP activity in biofilm formation is consistent with the knowledge that this peptide must be involved in processes other than natural genetic transformation, since only 23 of the more than 120 genes it induces in *S. pneumoniae* are needed for genetic competence. Another process that has been linked to CSP is termed competence-induced cell lysis. This activity, which involves production of the murein hydrolases LytA, LytC, and CbpD, has been viewed as an autolytic mechanism for DNA release. However, Leiv Håvarstein (Norwegian University of Life Sciences, Aas, Norway) reported new evidence to suggest that the actual mechanism may involve targeting of noncompetent cells by the competent fraction in a population (i.e., fratricide rather than suicide). Håvarstein and colleagues revisited a report that described an unusual cell aggregation phenotype in competent pneumococci, but not in parallel cultures of noncompetent cells (68). They found that this phenotype is a consequence of DNA release and that it is eliminated in a *cbpD* mutant or in a *lytA-lytC* double mutant. They also discovered a CSP-induced immunity protein, ComM, which somehow guards competent cells against the actions of their own lysins. This system is postulated to provide competent pneumococci with specialized weaponry to acquire transforming DNA and nutrients while destroying noncompetent competitor cells in the immediate environment (30).

VACCINES AND ANTIMICROBIALS

The prominent role of *L. lactis* in industrialized food fermentations has fueled worldwide interest in the molecular biology of this species for decades. The knowledge base assembled from that work has provided greater control over industrial processes that utilize this microbe and has also stimulated interest in more novel applications. One of the most exciting and promising new applications involves the use of lactococci and nonpathogenic streptococci as live vectors for the delivery of vaccines or therapeutic proteins to mucosal surfaces (71). Historically, attenuated variants of pathogenic bacteria attracted the greatest interest for vaccine development, but nonpathogenic species offer an additional level of safety, and some

possess innate adjuvant activity (50). Gianni Pozzi (University of Siena, Siena, Italy) outlined the advantages of mucosal targeting with live vaccines and reported on experiments with *S. gordonii*, a human commensal bacterium, as a host for surface display of a candidacidal single-chain antibody. Pozzi found that the recombinant strain was as effective as the best antifungal drug (fuconazole) in clearing *Candida* infection in a rat vaginal model, and the work indicated that live agents also provided greater duration of control activity than traditional topical medicines.

The efficacy of lactococci as live vaccines was reinforced in a presentation by Immaculada Margarit (Chiron Vaccines, Siena, Italy), who described work with lactococci engineered to express pilin-like products from *S. agalactiae* (GBS). Pili are proteinaceous filaments that facilitate host colonization by virtue of their adhesive function. Once thought to be largely confined to gram-negative species, pilin-like structures and genes encoding their production have recently been described in gram-positive pathogens, including GBS and *S. pyogenes* (GAS) (39, 46). These structures have exciting potential as candidates for new vaccines, because they are recognized by serotype-specific antibodies, and mouse model studies have shown that vaccines comprised of recombinant pilus proteins confer protection against mucosal infection with GAS or GBS (46, 58). These results are very promising, but concerns exist that the effectiveness of protein vaccines may ultimately be limited by the broad variation in pilus composition that exists among strains. Margarit and coworkers showed that *L. lactis* can be engineered to produce hybrid pili with protective antigens from two different GBS and that the recombinant lactococci conferred protection against GBS lethal challenge on either systemic or mucosal immunization (12). Further exploitation of this system will determine whether lactococci can function as a multivalent live vaccine against a broad range of streptococcal diseases.

Lactococcal drug delivery systems can also be utilized in therapies that do not involve specific microbial pathogens or disease. Lothar Steidler (Alimentary Pharmabiotic Centre, Cork, Ireland) delivered an update on his work to combat human inflammatory bowel disease using *L. lactis* strains engineered to express interleukin 10 (IL-10) and other biologically active proteins. Lactococci are able to synthesize and process fully active IL-10, and previous work in a mouse colitis model showed that these cells prevented the onset of new disease and improved the histological condition of tissue in mice with existing colitis (63). The lactococcal IL-10 construct (LLIL-10) has now been tested in phase I human clinical trials with 10 individuals suffering from Crohn's disease. The patients showed no adverse reactions to the bacterium, and clinical efficacy was indicated by a reduction in the levels of C-reactive protein (10). Steidler also presented promising information on potential treatment of food allergens through LLIL-10 administration. The incidence of food allergies is rising in Western countries, and concern over contamination with common allergens is a leading cause of food recalls in the United States. Interestingly, administration of LLIL-10 was found to reduce the severity of anaphylaxis in mice made allergic to the milk protein β -lactoglobulin, offering hope for future treatment of this condition in humans.

The vision of utilizing nonpathogenic lactococci, strepto-

cocci, or enterococci as hosts for live vaccines and drug delivery systems is almost certainly rooted in the historic recognition that these organisms produce a variety of compounds that inhibit pathogenic bacteria. Some of the most potent antimicrobial agents produced by these cells are bacteriocins, proteinaceous compounds that may exhibit very broad spectra of inhibition. The significance of bacteriocins in food preservation continues to spur international inquiries into their nature, genetics, and modes of action (49). Ingolf Nes (Norwegian University of Life Sciences, Aas, Norway) described the activity and genetics of bacteriocins produced by enterococci, which are collectively termed enterocins. Bacteriocin production is particularly abundant among enterococci, and while most are class II agents, class I and class III enterocins have also been recognized. The prevalence of enterocin production may be linked to the finding that genes for many of these antimicrobials are carried on plasmids and frequently lie in close proximity to transposons or transposon-like elements. Interestingly, many enterococci produce cocktails (two to at least four) of different bacteriocins. In fact, 77% of 35 enterococcal strains recovered from the feces of breast-fed infants produced more than one bacteriocin. Though the biological significance of this observation is still undefined, its prevalence and diversity suggest that bacteriocins have an important role in cellular physiology.

THE EIGHTH STREPTOCOCCAL GENETICS MEETING

As is evident from this brief overview of the meeting highlights, the future of streptococcal genetics holds tremendous promise. Not captured in this article are the nearly 250 poster presentations we were able to enjoy at the meeting. These posters displayed the dedication and insight of a group of talented young investigators vigorously attacking the gaps in our present knowledge of streptococcal genetics, biochemistry, physiology, and pathogenesis, and a number of these presentations have been published in this issue of the *Journal of Bacteriology*. Clearly, the field is growing and thriving. We impatiently await the Eighth International Conference on the Genetics of Streptococci, Lactococci, and Enterococci, which is tentatively slated for 2009.

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AUTHOR'S CORRECTION

The Seventh International Conference on the Genetics of Streptococci, Lactococci, and Enterococci

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Volume 189, no. 4, p. 1209–1218, 2007. Page 1210, column 2, first complete paragraph, the last three sentences should be replaced with the following: “Newly appearing type 19A strains account for most of the increased antibiotic resistance now seen within the serotype. Work performed by Beall’s collaborator, Angela Brueggemann, depicted how a new invasive type 19A variant appears to have arisen following horizontal transfer of a 39-kb DNA fragment containing serotype 19A capsule and flanking penicillin-binding protein genes from an ST199 donor strain to a serotype 4 ST695 recipient strain. Serotype 4 capsular polysaccharide corresponds to one of the seven vaccine serotypes successfully targeted by PCV7. This single genetic replacement event appears to account for the rapid emergence of this vaccine escape 19A variant on the ST695 background.”