JOURNAL OF BACTERIOLOGY, Apr. 2008, p. 2649–2662 Vol. 190, No. 8
0021-9193/08/$08.00+0 doi:10.1128/JB.01950-07

MEETING REVIEW

Pseudomonas 2007[▽]

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Pseudomonas 2007, the Eleventh International Congress on Pseudomonas, was organized by the American Society for Microbiology and was held at the Westin Seattle in Seattle, Washington, 26 to 30 August 2007. Three hundred thirty participants attended this meeting from 30 countries. There were 27 invited speakers. There also were 220 posters, among which 22 were chosen for oral presentations. The meeting highlighted the remarkable breadth of the field while emphasizing the similarities and differences between species in the genus *Pseudomonas*. Some of these exciting developments are summarized in this review of the talks presented at the meeting. With a few exceptions, the presentations are grouped according to the sessions in which they were presented. In addition, in this issue of the *Journal of Bacteriology*, a number of papers provide in-depth coverage of some of the oral and poster presentations from the meeting.

KEYNOTE SPEAKER

Pseudomonas is one of the most cited bacteria in Medline. There are many possible reasons for this. *Pseudomonas aeruginosa* is the third most common nosocomial pathogen in our society and is associated with chronic and eventually fatal lung disease in cystic fibrosis (CF) patients, while *Pseudomonas syringae* species are prominent plant pathogens. The fluorescent pseudomonads are exceptionally nutritionally versatile, and this extends to unusual and toxic chemicals, making them one of the most important organisms for understanding biodegradation, for developing bioremediation measures, and for key industrial biotransformations. In addition, they are widespread in the environment but relatively well conserved compared to many other species. These characteristics have driven researchers to sequence many *Pseudomonas* genomes and develop an impressive array of genetic and functional genomic tools (including three *P. aeruginosa* genomic mutant libraries), which makes pseudomonads particularly amenable to investigation. Perhaps because of these characteristics, the keynote speaker, Maynard Olson (University of Washington), boldly predicted, "*Pseudomonas* will play a leading role in the direction biology will go."

Olson, the "father" of the first *Pseudomonas* sequence (*P. aeruginosa* PAO1), led off the conference by asking three basic questions. (i) What is *Pseudomonas aeruginosa*? (ii) What new possibilities exist for studying *Pseudomonas* in real-world environments? (iii) What can basic scientists do to ameliorate the suffering caused by *Pseudomonas aeruginosa*? The first question was answered by reference to classical microbiology and molecular sciences. Olson reported that this organism had a very substantial, highly conserved core genome involving as much as 90% of the genome but that the remaining, less-conserved regions of the genome showed substantial variability. For example, environmental isolates from within a single Belgian river (81) or isolates from patients with similar diseases were remarkably diverse, showing a few major groupings of strains but no dominant strain correlating with habitat or phenotype and very few population-specific single nucleotide polymorphisms (116). The second question was answered with reference to sequential isolates from a CF patient. Although only 68 mutations were identified in the 4× coverage shotgun sequence of isolates obtained years apart from a single patient's sputum (101), the indication from the ratio of synonymous to nonsynonymous mutations was that *Pseudomonas* was under strong positive selection, consistent with the many observed sequence alterations in virulence genes. For the third question, it was pointed out that access to enhanced functional genomic tools in *Pseudomonas* is facilitating major advances in the understanding of tobramycin sensitivity and growth of this organism in patients, which Olson suggested would have a significant impact on patient treatment. Concluding, Olson made an impassioned plea for basic scientists to become aware of their ability to address translational goals.

SIGNAL TRANSDUCTION AND GENE REGULATION

Udo Blaesi (University of Vienna) explained the importance of small noncoding RNAs (ncRNAs) in posttranscriptional regulation of gene expression, including in *Pseudomonas*. These ncRNAs can bind to the target mRNA and either activate or prevent translation by changing their conformation. Hfq is an RNA chaperone that binds ncRNAs and contributes to their stabilization or joins ncRNAs and target mRNA (109). In *P. aeruginosa*, the ncRNAs RsmZ/RsmY and PrrF1/PrrF2

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▽ Published ahead of print on 28 December 2007.

have been described (53, 117), and RsmY was shown to bind specifically to Hfq (102). Seventeen novel ncRNAs were predicted by using a bioinformatics approach, and two of them, PhrS and PaeIII, were further investigated. PhrS, found between PA3305 and PA3306, is produced in stationary phase and binds Hfq. Proteome analysis revealed that *phrS* overexpression caused an increase of the chaperone GroEL and of the specialized porin OprD. The gene *mvfR* (also called *pqsR*) was predicted and confirmed to be a PhrS target, adding another regulatory level to the production of the *Pseudomonas* quinolone signal (PQS). The second RNA, PaeIII, between PA3535 and PA3536, is also produced in stationary phase. A proteomic analysis revealed PA1202 (a probable hydrolase of the isochorismatase family, dependent on the extracytoplasmic function sigma factor PvdS) to be a target. Overexpression of PaeIII caused an increased expression of type III secretion genes, the *mexXY* efflux genes, and the *mexZ* gene, while causing a decreased expression of genes for arginine metabolism.

Dieter Haas (University of Lausanne) presented data on RsmA, a protein that binds some mRNAs in their 5′ untranslated leader, often at or near the ribosome binding site (RBS), resulting in inhibition of translation initiation. RsmA is titrated by small RNAs containing multiple GGA (RBS-like) motifs in unpaired regions. Production of these small RNAs is under control of the GacA response regulator in pseudomonads. In the plant growth-promoting *Pseudomonas fluorescens* strain CHA0, the two-component system GacS/GacA controls the expression of three RNAs, RsmX, RsmY, and RsmZ, which are antagonists of the corresponding translational repressors RsmA and RsmE. A triple *rsmXYZ*-negative mutant produces no antifungal antibiotics and has consequently lost its biocontrol activity (52). A motif highly conserved in GacA-dependent promoters is present upstream of the RNA genes. The nature of the signal recognized by GacS is unknown, but it is present in the supernatants of different pseudomonads and even in *Vibrio* spp. Another sensor, RetS, is an antagonist of the GacS/GacA cascade (37). Finally, analysis of the 5′ region of *hcnA* mRNA reveals that the RsmA or RsmE dimers bind to three sites, one of which includes the RBS (55, 96). A consensus binding site ([U/A]CANGGANG[A/U]) was derived and substantiated by structural data (96).

In *P. aeruginosa*, the transition from the planktonic to the biofilm mode of life is mediated by regulatory switches (37). Planktonic cells are more prone to cause acute disease, while biofilms cells are responsible for chronic disease. As in *P. fluorescens*, the GacS/GacA two-component system promotes the transcription of two small RNAs, RsmZ and RsmY, which regulate the production of secondary metabolites, promote the biofilm mode of growth, and repress the expression of type III secretion. Stephen Lory (Harvard Medical School) focused on an important secondary messenger in the cell, cyclic di-GMP (c-di-GMP), which is synthesized from GTP by proteins having the GGDEF motif. Conversely, proteins containing the EAL motif degrade c-di-GMP. Elevated levels of c-di-GMP cause a decrease in toxin production and an increase in the production of extracellular matrix polysaccharides (*pel*, *psl*, and *alg* genes). One of the proteins of the Pel polysaccharide biosynthetic machinery (PelD) is a receptor for c-di-GMP (59). The above-mentioned sensor RetS is important because it antagonizes RsmA activity, which itself represses the translation of mRNA of the *pel* operon. Another c-di-GMP receptor, Alg44, is a transmembrane adaptor protein that mediates the regulatory function of this dinucleotide during synthesis of the alginate polysaccharide (68). Important questions for the future are those regarding the mechanisms of c-di-GMP-mediated regulation, the signals that regulate c-di-GMP synthesis/degradation, and the explanation for the redundancy of GGDEF/EAL proteins.

Simon Dove (Boston Children's Hospital) presented data about the regulation of the *cupA1* to *cupA5* genes, which are involved in biofilm formation in *P. aeruginosa* (110). The small protein MvaT represses a phase-variable expression of *cupA* genes, and its absence is thought to result in a mixed population of fimbriated and nonfimbriated cells (111). A genetic screen revealed three positive regulators of *cupA* gene expression, encoded by the genes PA2126 and PA2127, which are upstream of *cup*, and by a putative ORF, PA2126.1, which overlaps these two genes (112). These genes were named *cgrA*, *cgrB*, and *cgrC* (for *cupA* gene regulator). The anaerobiosis regulator Anr is a fourth positive regulator of *cupA* genes, which are expressed under anaerobic conditions but not when the *cgr* genes are deleted. Likewise, *cgr* gene expression is enhanced by anaerobiosis. Chromatin immunoprecipitation confirmed the binding of Anr to the *cgr* promoter region. This means that Anr is the prime activator of *cupA* gene expression, first increasing the expression of the *cgrABC* genes, which in turn up-regulate *cupA* expression.

Transcriptome mapping, presented by Melanie J. Filiatrault (USDA Agricultural Research Service), is a new approach which consists of isolating mRNAs after removal of rRNAs, converting them into cDNA, subjecting them to shotgun sequencing using pyrosequencing (high-throughput 454 technology), and comparing them with the annotated genome. This approach allows the discovery of small RNAs in noncoding regions and previously undiscovered genes (not annotated) and identification of transcription start sites and was used to analyze the global transcriptome of the plant pathogen *P. syringae* DC3000. After filtering of nonunique and rRNA sequences, a total of 7,786 reads were assembled into 3,597 "containers." Of these containers, 1,849 mapped to known coding sequences (CDS) (32%), 437 to hypothetical CDS (27%), 297 to noncoding regions, and 788 to CDS and intergenic regions. Of 54 genes that were previously found to contain a putative HrpL promoter site in their promoter region (27), 17 were found to have a putative transcriptional start site located in proximity to a predicted HrpL promoter site, providing additional evidence that these are functional promoters. Also, expression of six small RNAs (www.sanger.ac.uk/Software/Rfam) was confirmed, and several candidate small RNAs were identified. Undoubtedly, posttranscriptional regulation by small RNAs in pseudomonads is a field that will bring very interesting discoveries to light.

Quorum sensing (QS) regulates many virulence and colonization traits in *P. aeruginosa*, and its regulation is highly complex and hierarchical (48, 58, 113). The talk given by Giordano Rampioni (University of Rome) centered on a regulator of QS in *P. aeruginosa*, RsaL. The *rsaL* gene is between the *lasR* and *lasI* genes, which encode a LuxR regulator and the 3-oxo-C_{12}-homoserine lactone (HSL) synthase, respectively, both important components of the QS circuitry of *P. aeruginosa*. In an

rsaL mutant, 3-oxo-C_{12}-HSL production is highly enhanced with respect to the wild-type strain (86). RsaL is a helix-turn-helix protein that binds simultaneously with LasR to the *rsaL-lasI* bidirectional promoter, thereby preventing the LasR-dependent activation of both genes (87). Thus, RsaL functions in opposition to LasR and limits 3-oxo-C_{12}-HSL production. Furthermore, transcriptional profiling revealed that RsaL regulates 130 genes independently of its effect on QS signal molecule production. RsaL binding to the promoter regions of the QS-regulated genes *phz* and *hcn* was confirmed, and RsaL was also found to bind to the region upstream of a regulator encoded by PA2115 (88).

The third quorum-sensing signal, PQS, is one of several characterized QS systems that also controls virulence factors in *P. aeruginosa*. Everett Pesci (East Carolina University) described the biosynthesis of PQS through condensation of a fatty acid and anthranilate, with the latter being supplied from two sources: the kynurenine pathway and PhnAB anthranilate synthase (26). The redundancy of pathways providing essential precursors for PQS synthesis indicates that the production of this signal molecule is critical to the fitness of *P. aeruginosa* and that the PQS biosynthetic pathway may serve as an excellent target for the development of novel therapeutics against this bacterial pathogen.

Nelli Boes (Technical University, Braunschweig, Germany) talked about universal stress proteins. Universal stress proteins are ubiquitous, and five of them are expressed in *P. aeruginosa* under anaerobic conditions. Among them, UspK is important for pyruvate fermentation and UspN for survival in stationary phase under anaerobic conditions (13, 95). The global regulator of anaerobic metabolism Anr (mentioned above as a regulator of *cup* genes) controls the transcription of *uspN* via binding to an Anr box under anaerobic conditions. Induction of *uspN* transcription in stationary phase (Anr independent) is mediated by the stringent response via the level of ppGpp and the RelA and SpoT proteins. No transcription of *uspN* occurs in a triple *anr relA spoT* mutant.

PSEUDOMONAS AND ITS HOSTS

Burkhard Tümmler (Medizinische Hochschule Hannover, Hanover, Germany) has been performing genome-wide analyses of *P. aeruginosa* by microarray genotyping of the core and accessory genes of *P. aeruginosa* strains. It was found that 15 clones made up 50% of the population. The most frequent genotype was represented by the sequenced strain PA14 (116), while PAO1 is part of a clone that is found relatively frequently, and strains 2192 and PAK represent single clones.

CF is caused by molecular lesions in the *CFTR* gene. The age-dependent risk of the acquisition of *P. aeruginosa* can be differentiated by the *CFTR* mutation genotype. In a European CF twin and sibling study which searched for genetic modifiers of CF, loci of the innate immune system were found to modulate the susceptibility to and severity of chronic *P. aeruginosa* infections in CF. The CF lung is an atypical niche influenced by variable selective pressure for *P. aeruginosa*, leading to microevolution. In the absence of nosocomial transmission and antimicrobial pressure, the distribution of clones in CF lungs matches that in other clinical and environmental habitats. Prognosis of *P. aeruginosa* lung infection correlates with clone and clade. The initially colonizing clone persisted on the average for about 20 years in the patient's airways, where it acquired a habitat-specific phenotypic signature, involving attenuation of motility, QS, or virulence. Adaptive radiation in the accessory genome of *P. aeruginosa*, however, was highly variable between CF hosts. Divergent evolutionary lineages derived from the same ancestor in unrelated CF patients showed substantial differences in their transcriptomes and proteomes upon exposure to inanimate stresses and mammalian host defenses.

Laurence Rahme (Massachusetts General Hospital) has been studying different model systems to identify factors involved in host-pathogen interactions (4, 56, 76). At this meeting, she discussed results obtained with *Drosophila* infection as a model system to identify host components and pathways that can affect the initiation and progression of *P. aeruginosa* infection after wounding. Using two different *P. aeruginosa* strains, she found that flies were more susceptible to infections with strain PA14 than strain CF5 (2). It was demonstrated that 241 genes were differentially expressed in flies infected with these strains. Among these genes were those involved in pathogenesis and defense, including those involved in antimicrobial peptide and skeletal muscle protein expression. The latter genes were investigated more fully using flies that were deficient in the c-Jun N-terminal kinase (JNK) pathway. These flies were more susceptible to localized infection, suggesting a role for this pathway in protection from infection (3).

In contrast to using *Drosophila* as a surrogate host to model *P. aeruginosa* infections in humans, Isabelle Vallet-Gely (CNRS-CGM) uses a species of *Pseudomonas* that is a true pathogen for this insect. *Pseudomonas entomophila* is highly pathogenic for insects, leading to persistence through evasion of the immune response and the resulting massive destruction of gut cells. The genome sequence of this species has been determined. Interestingly, there is no type III secretion system or associated toxins. However it was demonstrated that this species contains potential virulence factors that could contribute to pathogenesis, including insecticidal toxins, proteases, lipases, hydrogen cyanide, lipopeptides, and certain secondary metabolites. A genome-wide random mutagenesis screen identified the well-studied two-component regulator GacS/GacA by its ability to regulate most of these potential virulence factors. AlgR was also required for evasion of the immune response. Directed mutagenesis was also performed, and an AprA protease was recognized. This protease was controlled by GacA but did not show as strong a defect in virulence as did the *gacA* mutant. However, both the AprA mutant and the wild-type strain survived in the absence of an immune response in the fly, suggesting that this protease may protect against antimicrobial peptides (60). A nonribosomal peptide synthetase gene was also directly inactivated. This mutant persisted in the fly gut but was impaired in its pathogenic properties.

Coinfections with *Candida albicans* and *P. aeruginosa* have been shown to be medically important in opportunistic infections, such as those on medical implants, in ocular infections, on the skin during burn infections, and in respiratory infections in CF patients. About 75% of the human population is colonized with *C. albicans*. Deborah Hogan (Dartmouth Medical School) presented studies showing that the *P. aeruginosa* au-

toinducer 3-oxo-C_{12}-HSL represses hyphal growth of the fungus (46) and that *P. aeruginosa* can rapidly colonize and kill *C. albicans* hyphae. *P. aeruginosa* phospholipase C contributes to the killing of *C. albicans*. In a screen to look for additional mutants with altered interactions with *C. albicans*, a novel regulator encoded by PA5380 was found. The PA5380 mutant could not grow on choline or phosphatidylcholine catabolic products, including glycine betaine and dimethylglycine. PA5380, named GbdR (for glycine betaine-, dimethylglycine-responsive regulator), encodes an AraC-like transcriptional activator that controls expression of nearby genes responsible for glycine betaine utilization. This transcription factor also controls the expression of the hemolytic phospholipase C. Follow-up experiments indicated that GbdR controlled the expression of genes important for not only the interactions between *P. aeruginosa* and *C. albicans* but also the interactions between *P. aeruginosa* and human epithelial cells.

For chemical screening, a whole-organism infection model is desirable. Such a system should allow the detection of candidates involved in virulence and gene products essential for in vivo growth, as well as making it possible to monitor the effects of these molecules on the host. Anne E. Clatworthy (Massachusetts General Hospital, Harvard Medical School, and the Broad Institute) presented the use of zebrafish embryos as a model for this approach. These embryos are amenable to screening in 96-well plates. Zebrafish are jawed vertebrates and as such have well-developed innate and adaptive immunity similar to that of mammals (106), although embryos possess only innate immunity, including both macrophage and neutrophil cell populations (9). In addition, zebrafish are genetically tractable, and the embryos are transparent, facilitating the visualization of infection as it progresses. Given their advantages as a model organism, they were considered as a host for *P. aeruginosa* infection, and an attempt was made to infect embryos by static emersion in PA14. Unfortunately, only a high concentration of PA14 (10^9 CFU) exhibited a lethal phenotype, but this lethality was independent of whether the bacteria were viable, in that heat-killed bacteria were equally lethal. Therefore, an attempt was made to establish a lethal *P. aeruginosa* infection in embryos by microinjection of bacterial cells directly into the circulatory system. It was found that microinjection of >2,500 CFU of strain PA14 resulted in a lethal phenotype, unlike microinjection of either heat-killed PA14 or similar numbers of *E. coli* DH5α cells. Interestingly, embryos at different developmental stages had different susceptibilities to infection with *P. aeruginosa* mutants. Embryos infected at an early developmental stage, i.e., 28 h postfertilization (hpf), were equally susceptible to infection with PA14 and that with *lasR* and *mvfR* mutants. In contrast, the *lasR* and *mvfR* mutants were attenuated for infection of embryos at a later developmental stage (48 hpf). Interestingly, the observation that the *lasR* and *mvfR* mutants are attenuated only in later-stage embryos correlates with the appearance of neutrophil activity in the developing embryo. Functional neutrophil activity (61), as measured by the migration to sites of injury and infection, is detectable 48 hpf, whereas macrophage clearance of invading microorganisms is detectable far earlier (26 to 30 hpf) (43), indicating that differences in zebrafish immunocompetence at 28 hpf and 48 hpf may underlie differences in susceptibility to infection with the *lasR* and *mvfR* mutants. In addition to host developmental-stage-specific factors, the zebrafish genetic background also contributed to the progression to lethal infection, in that a zebrafish line that was less susceptible to PA14 infection was identified. Finally, it was demonstrated that embryos could be rescued from the lethality of infection with antibiotics, further establishing the feasibility of screening small molecules that attenuate infection. While perhaps not as useful for extremely high-throughput analyses, this makes this system an attractive model for monitoring *P. aeruginosa* mutants and screening compounds to inhibit *P. aeruginosa* infections.

Roger C. Levesque (Université Laval) presented in vivo growth data using a rat model of chronic infection for four strains of *P. aeruginosa* (54a). In this model, 10^6 CFU was added to agarose beads. Strains were compared for infection kinetics, bacterial persistence, and liberation and localization within the tissues of the lungs. Two weeks after infection, all of the strains had similar growth rates in vivo. The hypervirulent Liverpool epidemic strain LESB58 did not move out from the initial site of infection, while PAO1, PA14, and PAK disseminated and were found in the alveolar regions. LESB58 was found to have growth defects on minimal medium but made larger biofilms than the other strains. This strain also had swimming and twitching motility defects. In terms of competitiveness, the assessment of competitive indices indicated a relative ability to persist: PAO1 > LESB58 > PA14.

CELL-TO-CELL COMMUNICATION

Robert Hancock (University of British Columbia) described bacterial swarming, a concerted type of bacterial motility on semisolid surfaces, which is mediated by flagella and type IV pili in pseudomonads (74a). A transposon *luxCDABE* fusion library of mutants was generated and swarming-deficient mutants selected (75). Many different types of mutants in various categories were obtained, including some affected in general metabolism, indicating that swarming was a complex adaptation rather than just a method for movement. Flagellum-deficient mutants could not swarm or swim but could twitch (pilus mediated), while pilus-deficient mutants could swim (flagellum mediated) but did not swarm or twitch. A microarray approach was used to compare swimmer versus swarmer cells, revealing a number of up-regulated (309) and down-regulated (108) genes. Swarming cells at the edges of the swarming community showed higher expression of genes corresponding to type III secretion, different virulence factors, phenazine biosynthesis, pyochelin and pyoverdine siderophore biosynthesis, the MexGHI-OpmD efflux pump, and alkaline protease. The importance of pyoverdine for swarming motility was confirmed by the inability of a mutant with a mutation in *pvdQ*, which codes for an acylase and is involved in pyoverdine biosynthesis, to swarm (100). This would appear to confirm the dual function of pyoverdine as a siderophore and as a signal molecule. QS was also important, since *rhlI* mutants were unable to swarm. Type II secretion was necessary as well, and in particular two secreted proteins, LasB and protease IV. Mutants with mutations in the Lon protease were defective both in swarming and in biofilm formation (63) and were hypersusceptible to fluoroquinolones. The TetR regulator PsrA was important as well for swarming and for resistance to polymyxin. In conclusion, these

results show that there is an overlap between swarming, biofilm formation, QS, and adaptation to antibiotics.

Paul Williams (University of Nottingham) presented data on the small protein RsmA (see also the discussions of talks by U. Blaesi and D. Haas in Signal Transduction and Gene Regulation), which negatively regulates the production of protease, elastase, cyanide, lectin, and the blue-green pigment pyocyanin in *P. aeruginosa*. As in *P. fluorescens* CHA0, two small RNAs, RsmZ and RsmY in *P. aeruginosa*, bind RsmA and block its function as a translational repressor (45). Conversely, RsmA had a positive influence on swarming and on the production of rhamnolipids and lipase (45). RsmA also exerted a transient repression on the expression of *lasI* and *rhlI*, which encode the two *N*-acyl-HSL synthases of *P. aeruginosa* (80). PQS activated *rsmZ* in a dose-dependent manner and was one, but not the only, signal inducing the expression of this gene. It was found that Arg44 is an essential residue for the binding of RsmA to *rsmZ* (39). Pull-down experiments were performed using His-tagged RsmA to identify the target mRNAs. RsmA was found to interact with its own transcript as well as with mRNAs encoding regulators and genes involved in secondary metabolism and QS (such as *lecA*). Six of the transcripts corresponded to genes involved in c-di-GMP metabolism. One such gene, PA2567, encodes a protein with GAF-GGDEF-EAL domains and in vitro functions as a c-di-GMP phosphodiesterase (93). An *rsmA* mutant could not swarm, but swarming could be restored by expression in *trans* of PA2567 and other c-di-GMP phosphodiesterases (93), indicating that *rsmA* mutants may accumulate c-di-GMP and highlighting the importance of the secondary signal molecule c-di-GMP for the social motility of *P. aeruginosa*.

Another important pseudomonad, *P. syringae*, which can cause both disease on plants and frost damage via its ice nucleation capacity, was the subject of the talk given by Steven E. Lindow (University of California, Berkeley). *P. syringae* lives in aggregates of various sizes on leaves, and while relatively few of these are composed of more than 100 cells, some can be very large; aggregates larger than 100 cells represent a large proportion of the total population. QS in *P. syringae* depends on the LuxR regulator AhlR, on the AHL synthase AhlI, and on another regulator, AefR (83, 84). QS is necessary for survival under dry conditions and for the expression of the extracellular polysaccharide (EPS). In contrast to the situation with *P. aeruginosa*, QS suppressed swarming, and QS mutants invaded leaves more quickly. Other bacteria can influence QS of *P. syringae* positively or negatively. Those stimulating QS in *P. syringae* produce the same AHL, 3-oxo-C_6-HSL, and reduce infection. Conversely, bacteria that inhibited QS increased the disease index. Finally, there was a link between QS and iron, since addition of ferric salts caused an increase in AHL production while tannic acid, an iron chelator, inhibited QS (51). In conclusion, both iron and QS modulated the fitness and the virulence of *P. syringae*.

QS can also be a burden for *P. aeruginosa*, as discussed by Martin Schuster (Oregon State University). Several hypotheses have been formulated to explain the relatively high proportion of spontaneous mutations in the *lasR* gene, which encodes a master LuxR-like regulator of the *P. aeruginosa* QS system: (i) QS is not important; (ii) *lasR* mutants resist alkaline lysis after cessation of growth (44); (iii) *lasR* mutants grow better on some carbon and nitrogen sources (19); and (iv) *lasR* mutants are social cheaters. Cheaters are at an advantage in mixed populations where cooperation is required. Experiments were done using a medium containing caseinate where only the wild-type strain can grow due to the production of QS-dependent extracellular proteases. After 100 generations, QS-negative mutants accumulated, reaching 60% of the population after 20 days. However, it was observed that compensatory mutations restoring protease production arose. All of the QS-negative mutants that were characterized further had a *lasR* mutation and could be complemented by *lasR*. Two mutants showed an increased production of C_4-HSL to compensate for the absence of LasR. Finally, it was found that QS genes are highly expressed when needed for growth (such as in the presence of caseinate) but not on other media, such as that containing Casamino Acids, thereby imposing a metabolic burden on the wild-type cell, which in turn provides a selective advantage for *lasR*-negative cheaters.

Modeling of the communication within spatially structured bacterial communities, such as *P. fluorescens* in the rhizosphere or biofilms formed by, e.g., *P. aeruginosa* in the CF lung, is possible, as shown by Andreas Dötsch (Helmholtz Center for Infection Research). Three factors can affect QS signaling: cell density, spatial distribution, and mass transfer (42). Cells inside a cluster (microcolony) were all activated simultaneously by signal molecules (such as AHLs), which was not the case for randomly distributed bacterial cells. In other words, microcolonies promote a social cooperative behavior. Microcolonies also insure the privacy of communication (listening only to its kin). It is postulated that autoinducer signaling within microcolonies and the positive feedback so generated were needed to maintain the privacy.

SECONDARY METABOLISM

The conference included a session devoted specifically to secondary metabolism, a striking characteristic of *Pseudomonas* spp., with talks focusing on siderophores, cyclic lipopeptides, phytotoxins, and antibiotics with antibacterial and antifungal activities. Pierre Cornelis (Vrije Universiteit Brussels, Brussels, Belgium) began the session with a discussion of siderophore-mediated iron acquisition systems in *Pseudomonas*. The fluorescent pseudomonads are characterized by their production of pyoverdine siderophores, which are responsible for the green fluorescence of these bacteria when cultures are viewed under UV light. Approximately 50 different pyoverdines have been isolated from different strains of *Pseudomonas* spp. (16). Specific TonB-dependent outer membrane receptors (termed FpvA and FpvB in *P. aeruginosa*) mediate the transport of cognate ferric pyoverdine complexes into the bacterial cell, and recently, FpvA was also shown to be the outer membrane receptor for the bacteriocin pyocin S2 (23). Many strains of *Pseudomonas* spp. also produce a second siderophore, such as pyochelin, pseudomonine, corrugatine, pyridine-2,6-bis-(thiocarboxylic acid), and the most recently identified compound, thioquinolobactin. Some of these siderophores have antimicrobial activity as well as a role in iron acquisition (65).

Many strains of plant-associated *Pseudomonas* spp. produce the antibiotic 2,4-diacetylphloroglucinol (DAPG), which is toxic for numerous plant-pathogenic fungi and contributes to

the biological control of plant disease (115). John Morrissey (University College Cork, Cork, Ireland) reported on studies evaluating the mode of action of DAPG in *Saccharomyces cerevisiae*, where DAPG interferes with mitochondrial function, most likely by targeting the F_1F_o ATPase. The group at the University College of Cork has also completed a genomic analysis of DAPG-producing strains, which indicated that the DAPG biosynthesis gene cluster is ancestral in *P. fluorescens*.

Phenazines are a class of more than 50 pigmented, heterocyclic nitrogen-containing secondary metabolites synthesized by some strains of fluorescent *Pseudomonas* spp. and a few other bacterial genera (66). The phenazines are of particular interest because they include pyocyanin, a virulence factor and signaling molecule of *P. aeruginosa* (24), as well as a spectrum of compounds with antifungal properties that are produced by plant-associated strains of *Pseudomonas* spp. Linda Thomashow (USDA Agricultural Research Service, Pullman, WA) shared recent results on the structural analysis of enzymes involved in the biosynthesis of phenazines. "Core" and "decorator" enzymes in the phenazine biosynthetic pathway have been purified and crystallized recently, allowing new insights into the biosynthesis of this important group of compounds and thereby suggesting strategies for interfering with the synthesis of this virulence factor. Krishna Maddula (University of Arizona) presented data on the role of phenazines produced by *Pseudomonas chlororaphis*, a plant growth-promoting rhizobacterium that controls take-all disease caused by the fungus *Gaeumannomyces graminis* (61a). The phenazines are responsible for the antagonism of this bacterium against the fungus. Phenazine production is regulated by the QS AHL synthase PhzI and the LuxR regulator PhzR and by the two-component systems GacA/GacS (positively) and RpeB/RpeA (negatively). Using a flow cell system, it was found that phenazines are also needed for biofilm formation (62). Two phenazines are produced by *P. chlororaphis*, phenazine carboxylic acid (PCA) and 2-hydroxy-PCA. The latter promotes initial attachment of cells on the surface, and changing the ratio of the two phenazines increases the thickness of the biofilm. Also, the dispersal rate of the biofilm was the highest for the wild-type strain and the lowest for the 2-hydroxy-PCA producer mutated in the *phzO* gene.

P. syringae secretes the toxin coronatine, which is an analog of jasmonates, a family of plant signaling molecules. This virulence factor plays a key role in the development of disease on plants. Mutants that are impaired in the production of coronatine or one of its components, coronafacic acid or coronamic acid, exhibit reduced virulence on plants (15, 107). Barbara Kunkel (Washington University) presented information on the use of both forward and reverse genetic approaches to investigate the role of coronatine during pathogenesis. Coronatine can promote pathogen entry into leaf tissue by stimulating the opening of stomata (67). Although coronatine-defective mutants are not able to multiply to high levels in wild-type *Arabidopsis* plants, the mutant strains grow to wild-type levels in *Arabidopsis* mutant plants that do not produce salicylic acid and are thus more susceptible to disease (14). However, even though the coronatine-defective mutants are able to infect these salicylic acid-deficient mutant plants, they still do not produce normal disease symptoms. These results indicate that coronatine is required for multiple steps in the pathway of *P. syringae* pathogenesis, including entry into host tissues, suppression of salicylic acid-mediated defenses, and promotion of disease symptoms.

Analysis of microbial genome sequences has revealed numerous orphan gene clusters, which have conserved sequences characteristic of secondary metabolism pathways but are not associated with known metabolic products. In *Pseudomonas* spp., the cyclic lipopeptides (CLPs) are the first secondary metabolites whose structures have been predicted from genomic sequence data and confirmed by chemical analysis (11, 21, 38). CLPs are synthesized by nonribosomal peptide synthases, and their amino acid composition can be predicted from the sequences of adenylation domains in the nonribosomal peptide synthase genes. Jos Raaijmakers (Wageningen University, Wageningen, The Netherlands) described recent developments in the bioinformatic predictions of CLP structures (20) and studies evaluating the physiological and ecological roles of this structurally diverse class of metabolites. All CLPs act as biosurfactants, and certain of them also exhibit antimicrobial or phytotoxic activities, function in biofilm formation or swarming motility (85), or contribute to biological control of plant disease (105). Joyce Loper (USDA Agricultural Research Service, Corvallis, OR) described the identification of metabolic products of orphan gene clusters from the genome of the soil bacterium *P. fluorescens* Pf-5 (77). In addition to the gene cluster specifying the biosynthesis of the CLP orfamide, another gene cluster is now known to specify the biosynthesis of analogs of rhizoxin, an antifungal and phytotoxic polyketide, and an insect toxin related to MCF (for makes caterpillars floppy). The genomic sequence of *P. fluorescens* Pf-5 has revealed new insights into the ecology and metabolic potential of this biological control organism which are currently being explored.

CELL SURFACES

The cell surfaces of gram-negative bacteria include an outer membrane, surface appendages, and occasionally additional extracellular protein or capsule layers (92). Studies have demonstrated an intrinsic role for cell surface molecules and structures in determining nutrient supply, pathogenesis, growth under nutrient limiting conditions, motility, exclusion of toxic materials, and the interaction of bacteria with other cells and surfaces.

Joe Lam (University of Guelph) described his latest research on the biogenesis of lipopolysaccharide (LPS) and generation of surface diversity. LPS is an unusual glycolipid, with a lipid A molecule inserted into the outer monolayer of the membrane and a long sugar chain (the variable core oligosaccharide capped or not by a repeating B-band O antigen or A-band unit) extending from the membrane. LPS contains the immunodominant O antigen and is also a major bacterial endotoxin, since the lipid A portion represents an important bacterial signature molecule that can stimulate innate immunity through interaction with TLR4. LPS also has a profound role in outer membrane barrier function. One ongoing puzzle is how this complex molecule, containing variable-sized oligosaccharide chains that can be one to hundreds of tri- to pentasaccharide O-antigen repeat units in length, is able to pass from the cytoplasm, where the units are synthesized, across the cytoplas-

mic membrane bilayer, while at the same time generating structural diversity. Lam described elegant biochemistry that permitted substantial breakthroughs in this area. The basic idea is that O-antigen chain units are assembled within the cytoplasm, transferred to a C_{55}-bactoprenol lipid carrier, flipped across the membrane by Wzx flippase, and then assembled into a polymer (Wzy) with the aid of a Wzz chain length-determining protein, before transfer onto core lipid A by WaaL (1, 36, 50). Further complexity can be provided by seroconverting sugar polymerases that require alternative flippases; indeed, such an arrangement was shown to explain the differences between the sugar linkages of serotypes O2, O5, and O16 despite similar sugar backbone compositions and O-antigen gene clusters.

In addition to LPS and the EPS alginate (which is associated with mucoidy in *P. aeruginosa*), there are two EPSs that have been recently shown to be involved in biofilm formation, namely, Pel and Psl (35). Dan Wozniak (Wake Forest University) provided an update on Psl, which is a mannose-rich polysaccharide produced by a 15-gene operon conserved in the fluorescent pseudomonads. Psl associates with the surface of *P. aeruginosa* in a unique helical pattern and can be observed very early in biofilm formation. Its distribution in biofilms changes throughout the biofilm cycle, and eventually it localizes on the periphery of biofilm microcolonies, a behavior which Wozniak interpreted as indicating that Psl may be a scaffolding device. Psl appears to confer protection against neutrophils, both by limiting the oxidative burst induced by opsonized bacteria and by protecting against certain reactive oxygen species. Intriguingly, there appears to be posttranscriptional reciprocal control of Psl and the EPS alginate, which is involved in mucoidy in CF isolates.

Marvin Whiteley (University of Texas) extrapolated from the fascinating observations of individuals like the recently deceased Terry Beveridge and sought mechanistic insight into the functions of membrane vesicles shed from the outer membranes of several bacteria, including *P. aeruginosa*. Such vesicles have been shown or proposed to have roles in interspecies communication (10), DNA transfer, and toxin antimicrobial delivery (22, 49, 64), and Whiteley discussed a new role in the secretion of quorum-sensing signaling molecules from cells. In particular, he indicated that PQS (79) was largely in the cell supernatant as vesicles (64), while the more traditional quorum-sensing signaling molecules, the *N*-acyl-HSLs, were predominantly free. Mutants (*pqsA*, *pqsH*) with defective PQS production made a very low level of membrane vesicles, and this could be complemented with chemically synthesized PQS. Detailed studies on the mechanism and structural basis provided evidence that PQS was able to stimulate acyl chain ordering in LPS, concomitantly decreasing membrane fluidity, and Whiteley thus proposed that PQS inserted into the outer membrane to stabilize areas of the outer membrane that were less closely associated with the peptidoglycan, thus causing membrane vesicle budding from the cell surface.

Barbara Kazmierczak (Yale University) described studies aimed at understanding assembly at the cell poles of motility organelles (flagella, which mediate swimming, and pili, which mediate twitching motility) (70a). She discussed how these two types of motility might be coordinately regulated through c-di-GMP metabolism and a series of proteins involved in the synthesis (diguanylate cyclase) and/or degradation (c-di-GMP phosphodiesterase) of this molecule. One of these proteins is FimX, which has GTP-activated phosphodiesterase activity (54). FimX mutants fail to assemble surface pili, resulting in altered colony morphology and the absence of twitching motility. Using a red fluorescent protein tag, FimX was demonstrated to localize primarily (75% of the time) to one pole. Thus, FimX might be a spatial cue for polar pilus assembly. A yeast two-hybrid system was used to identify four proteins that interact with FimX: PA0020, PA3670, PA4461, and FlhF. Overexpression of PA3670 (GldG), an ABC transporter, led to complete suppression of twitching, but pili still appeared on the bacterial surface. Intriguingly, PA3670 was localized to the pole, in a FimX-dependent manner. The envelope-associated protein PA0020 was also located at the pole, and deletion or overexpression of this gene decreased twitching. The most intriguing FimX-associated protein was FlhF. Deletion of FlhF resulted in the assembly of flagella on the lateral cell wall, rather than at the bacterial pole (70). In this background, normal suppression of the type IV pilus assembly during liquid growth failed to occur, suggesting a role for FlhF in this process. A model was presented in which FlhF might control FimX activity, and thereby pilus assembly, by modulating local GTP concentrations at the bacterial pole. A productive avenue for these studies will be to understand the basis for control of swarming motility, which requires both types of motility.

Juan Nogales (CSIC) described uptake of gallic acid by *Pseudomonas putida*, which is necessary for its degradation in vivo. He showed that the major transporter was GalT, an inducible major facilitator superfamily transporter that operates with the assistance of outer membrane protein (and possible porin) GalP. Lynn Wood (Virginia Commonwealth University) described the role of intracellular proteases in the cell wall stress stimulon. She showed that cell wall-active antimicrobials (β-lactams, phosphomycin, cycloserine, Tween-20, and triclosan) were able to induce the alginate operon as part of a cell wall stress regulon. She showed evidence that this included several regulators, including σ^{22} as well as two intracellular proteases, a YaeL homologue and AlgW (a DegS homologue). The implication was that β-lactam therapy, often used to control infections in CF patients, might assist or promote mucoid conversion.

Carmen Giltner, from McMaster University, discussed the role of five minor type IV pilins (the major one involved in pilus formation being PilA). Minor pilin mutants were immotile, but intriguingly, all of the minor pilins (FimU, PilV, PilW, PilX, and PilE) could complement this motility defect if expressed at a low level, with the exception of PilX, which inhibited wild-type twitching motility when overexpressed. Overexpression of two minor pilins, FimU and PilX, also increased type II secretion, a process with some overlaps with pilus extrusion and retraction. Thus, it appears that these minor pilins might have subtle roles in pilus assembly, surface expression, and, in two cases, type II secretion.

Membrane transport systems that facilitate the uptake of compatible solutes are important components of the osmotic stress response in many bacteria, and characterization of the first osmoregulatory transporter in *Pseudomonas* spp. was reported recently (17). Chiliang Chen (Iowa State University, Ames) provided an overview of ongoing studies evaluating

transporters in the ABC and BCCT (betaine/carnitine/choline transporter) families that function in osmoregulation in *P. syringae*. Characteristic cystathionine-β-synthase domains in the ABC transporters are required for osmoregulation, and their presence was used successfully to identify osmoregulatory transporters in the genome of *P. aeruginosa*. Choline, a compatible solute that is ubiquitous in plants, appears to be particularly important in osmoprotection of *P. syringae*, which possesses a choline transporter (BetT) that is rapidly activated by osmotic stress (18). Current studies in the Beattie lab (Iowa State University) are focused on the role of these osmoregulatory transporters in the ecology of *P. syringae*.

PSEUDOMONAS PHYSIOLOGICAL AND METABOLIC DIVERSITY

This session brought together a diverse set of talks on biodegradation pathways, enzymes, regulation, mechanisms of solvent resistance, and biotechnologically useful lipases. As the session progressed, unexpected connections to important aspects of *Pseudomonas* pathogenicity and QS emerged, as lipases are key virulence factors and enzymes in the quinoline degradation pathway were found to function in quorum quenching.

Susanne Fetzner (Westfalische Wilhelms-Universitat Munster) has been studying the degradation of quinoline and its derivatives by several species of bacteria (reviewed in reference 28). These compounds are widespread in the environment as naturally occurring plant materials, industrially important precursors of dyes, pharmaceuticals and antibiotics, and components of coal tar. The pathways for 1*H*-4-oxoquinoline and 2-methylquinoline degradation from *P. putida* 33/1 and *Arthrobacter nitroguajacolicus* Rü61, respectively, were found to have novel and very interesting ring cleavage dioxygenase enzymes, 1*H*-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (Qdo) and 1*H*-3-hydroxyoxoquinaldine 2,4-dioxygenase (Hod) (7, 29). The enzymes are members of the α/β hydrolase fold superfamily and are related to serine hydrolases (30, 82, 103). Both enzymes are small monomeric proteins (30 to 32 kDa), and neither has any cofactor requirements. Each incorporates two atoms from molecular oxygen at C-2 and C-4 of their respective substrates and releases an anthranilate derivative and carbon monoxide as products. Cleavage therefore occurs at two C-C bonds in the substrate (7). A catalytic mechanism involving ordered binding of substrate and deprotonation of the substrate by the conserved histidine residue at position 251, followed by oxygen binding and single electron transfer from the substrate to oxygen, has been proposed based on detailed studies of the purified wild-type and mutant forms of the protein. In this reaction scheme, the substrate is proposed to form a resonance-stabilized radical similar to that of flavin cofactors (34). Fetzner reported an exciting connection between Hod and degradation of gram-negative bacterial QS signals. PQS, which functions in both QS and iron entrapment (25), is structurally similar to an intermediate in the 2-methylquinoline pathway. Hod from the *Arthrobacter* strain was shown to interfere with QS by cleaving PQS to carbon monoxide and an anthranilate derivative. Thus, Hod is capable of quorum quenching and should be a useful tool in QS studies.

Dieter Jendrossek (University of Stuttgart) described recent progress in elucidating the pathways for catabolism of the acyclic monoterpenes citronellol and geraniol. These plant-derived aroma compounds have a methyl-branched structure, and to date the only microorganisms known to be capable of utilizing these compounds as carbon and energy sources include *Pseudomonas citronellolis*, *P. aeruginosa*, and *Pseudomonas mendocina* (32). In this study, the genes in the Atu (acyclic terpene utilization) and Liu (leucine/isovalerate utilization) pathways were analyzed in *P. aeruginosa*. Both citronellol and geraniol are oxidized to the corresponding carboxylic acids and then converted to coenzyme A (CoA) esters. The pathways converge at the common intermediate *cis*-geranyl-CoA following conversion of citronellyl-CoA to *cis*-geranyl-CoA by the action of AtuD (citronellyl-CoA dehydrogenase). A second enzyme with citronellyl-CoA dehydrogenase activity (encoded by PA1535) was identified, which may explain the appearance of suppressor mutants of an *atuD* knockout strain (31). *cis*-geranyl-CoA is difficult to degrade, because the presence of the β-methyl group inhibits β-oxidation. To circumvent this problem, the key enzyme geranyl-CoA carboxylase (AtuC/AtuF) carboxylates the β-methyl group. The requirement for AtuF was shown by the inability of an *atuF* mutant to grow on either substrate (47). The resulting carboxylated intermediate is then oxidized by the product of *atuE*, and acetate is released (possibly by the product of *atuA* [31]), thus eliminating the branch. Following two β-oxidations that release two molecules of acetyl-CoA, the intermediate 3-methylcrotonyl-CoA enters the Liu pathway, which is present in a wider range of bacterial species. Further degradation of this compound requires the elimination of a second methyl branch, which is catalyzed by 3-methylcrotonyl-CoA carboxylase (LiuB/LiuD) (47). Several of the *atu* and *liu* gene products were identified by two-dimensional gel electrophoresis following growth on linear terpenes, and the TetR family protein AtuR was found to be a repressor of the *atu* operon that responds to terpene effector molecules (31).

Hideaki Nojiri (University of Tokyo) discussed structure-function studies of carbazole 1,9a-dioxygenase, an enzyme that catalyzes the angular dioxygenation of the *N*-heterocyclic aromatic compound carbazole. The enzyme from *Pseudomonas resinovorans* CA10 has broad substrate specificity and can catalyze angular dioxygenation, lateral dioxygenation, or monooxygenation depending on the substrate provided (74). Carbazole dioxygenase is a member of the multicomponent Rieske nonheme iron oxygenase family. In addition to the catalytic oxygenase component, the enzyme requires specific electron transfer proteins to reduce the iron at the active site. The structures of the oxygenase (73), ferredoxin (71), and reductase were determined, as was an electron transfer complex between oxygenase and ferredoxin (5). When carbazole and oxygen were both bound in the active site, the substrate was positioned for angular dioxygenation, with C-1 and C-9a located closest to oxygen. Carbazole binding was stabilized by hydrogen bonding between the imino nitrogen and the main-chain carbonyl oxygen of G178. Several mutant forms of oxygenase with single amino acid substitutions at the active site were constructed and analyzed. In contrast to the wild type, a mutant form of the oxygenase with a Q282Y substitution produced primarily 1-hydroxycarbazole from carbazole. The crystal structure of the mutant protein indicated that carbazole was

positioned for lateral dioxygenation at C-1 and C-2. Spontaneous dehydration of the unstable carbazole *cis*-1,2-dihydrodiol could account for the production of 1-hydroxycarbazole. The results suggest that the active-site residues control regiospecificity by controlling the position of the bound substrate relative to the active-site iron and bound oxygen.

Linda Johansson (Umea University) presented a detailed analysis of the control of expression of the *dmpR* gene, which encodes the transcriptional activator of the *dmp* (phenol/dimethylphenol) catabolic operon in *P. putida* CF600 (reviewed in reference 99). Expression of the *dmp* catabolic operon from the promoter Po is activated by DmpR in the presence of pathway substrates and is also globally regulated through σ^{54}-RNA polymerase, the level of which is controlled by ppGpp and its coregulator DksA (10, 57, 104). In the absence of ppGpp, σ^{54}-RNA polymerase levels are low, effectively silencing Po regardless of the presence of inducing pathway substrates. As cells enter the stationary phase of growth, ppGpp levels increase in response to nutrient limitation. As a result, transcription from Po only occurs when pathway substrates are present and no preferred carbon sources are available. Using transcriptional fusions and Western blots, transcription from the *dmpR* promoter Pr was shown to occur at the exponential/stationary-phase transition, similar to transcription from Po. Expression from Pr was dependent on the presence of both ppGpp and DksA in vivo and in vitro, and occupancy of Po by σ^{54}-RNA polymerase was shown to stimulate expression from Pr. Together, these data suggest a model in which DmpR is autoamplified during expression from Po, resulting in a positive feedback loop that further maximizes production of the catabolic enzymes under appropriate environmental conditions.

Hermann J. Heipieper (UFZ Center for Environmental Research, Leipzig, Germany) provided an in-depth overview of the function and mechanism of *cis-trans* isomerase (Cti). This enzyme, which is found in *Pseudomonas* and *Vibrio* species, allows cells to rapidly adapt to solvent stress by converting unsaturated cytoplasmic membrane fatty acids from the *cis* to the *trans* form (reviewed in references 40 and 41). Unsaturated fatty acids are synthesized exclusively as the *cis* isomers, which do not pack tightly within the membrane because of the angle of the side chains and which therefore result in increased membrane fluidity. In contrast, saturated fatty acids and *trans*-unsaturated fatty acids pack tightly in the membrane; thus, membranes with higher saturated or *trans*-fatty acid contents are more rigid. While formation of saturated fatty acids requires de novo synthesis and occurs only in growing cells, *trans*-fatty acids are produced by enzymatic conversion of *cis*-fatty acids, and the reaction does not require growth, new enzyme synthesis, cofactors such as NAD(P)H, or input of energy. The enzyme responsible, Cti, is a periplasmic protein that is constitutively produced, and its activity correlates with stresses that increase membrane fluidity, including heat shock, organic solvents, osmotic shock, low pH, and antibiotic treatment. Cti has a conserved heme-binding motif, and the mechanism appears to involve removal of an electron by electrophilic iron from the *cis* double bond, followed by reformation of the double bond after rotation to the *trans* configuration (114). The current hypothesis regarding Cti activity is that upon any membrane insult that increases membrane fluidity, Cti gains access to fatty acid side chains from its location in the periplasm as a direct result of increased fluidity. As more *cis*-fatty acids are converted to *trans*-fatty acids, the membrane becomes more rigid and Cti access is then limited by the increased membrane rigidity. This rapid and self-limiting adaptation mechanism allows metabolically versatile bacteria like *Pseudomonas* and *Vibrio* to quickly adapt to changing environmental conditions. Measurement of *trans*/*cis*-fatty acid ratios has been suggested as a biomarker for monitoring stress.

Karl-Erich Jaeger (Heinrich Heine University Düsseldorf, Research Center Juelich) discussed the function and applications of two lipolytic enzymes from *P. aeruginosa*. LipA is an extracellular lipase produced by stationary-phase cells as a virulence factor, and it is also an important biocatalyst. Directed evolution using several random and directed mutagenesis methods ultimately resulted in a variant with high (*S*)-enantioselectivity that contained six amino acid substitutions (90). With the aid of the wild-type LipA crystal structure (72), molecular dynamics simulations were carried out to predict the roles of the six amino acids, and the main source of enantioselectivity was traced to the cooperative influence of two of the six mutations, namely, S53P and L162G (12). Based on this analysis, several single and multiple mutants were generated by site-directed mutagenesis, and it was shown that the double S53P L162G mutant was even more enantioselective than its parent with six mutations (89). The folding of active LipA involves the participation of the lipase-specific foldase Lif, which is encoded upstream of *lipA*. Lif is anchored to the cytoplasmic membrane by an N-terminal hydrophobic segment and faces the periplasm. LipA is produced as an inactive enzyme with an N-terminal signal sequence that directs the protein to the periplasm via the Sec pathway, where Lif ensures proper folding into an active conformation (91). The crystal structure of a LipA/Lif complex showed extended contact areas between the proteins (78); however, the mechanism of folding still remains elusive. Both genes could be expressed in a heterologous host and resulted in active lipase; in fact, Lif was shown to stimulate LipA folding in vitro (91). In contrast to LipA, which is secreted across the outer membrane by the type II (Xcp) secretion machinery, the *P. aeruginosa* esterase EstA is an autotransporter protein that is produced in the cytoplasm and self-secreted to the outer membrane. The catalytic N-terminal domain is folded into its active form outside the cell (119). A useful biotechnological application of EstA is the use of EstA fusion proteins to display and screen various enzyme activities (8). Recently, this system was used for functional cell surface display of the Lif chaperone and fluorescence-activated cell sorting-based analysis of bacterial cells that carried foldase-lipase complexes: this method now enables the ultrahigh-throughput screening of large libraries of foldase variants generated by directed evolution (118).

TYPE II AND TYPE III SECRETION AND TRANSPORT

Alain Filloux (CNRS-Marseille-France; at Imperial College London as of 2008), the organizer of the last *Pseudomonas* meeting, which was held in 2005 in Marseille, France, gave an overview of the type II protein secretion system that is present in most gram-negative bacteria. Type II secretion is one of the several molecular mechanisms allowing proteins to be trans-

ported across the outer membrane via a pore called secretin and it is powered by traffic ATPases. The type II secretion system is responsible for the release of most toxins, including elastase and exotoxin A. In this case, precursors are moved across the inner membrane in an unfolded (Sec-dependent) or folded (twin arginine translocase-dependent) state. There are two very complete type II secretion systems in *P. aeruginosa*, the QS-regulated Xcp system and the Hxc system, which functions under phosphate limitation. In some cases, the secretin of the Xcp system could be replaced by an orphan secretin called XqhA, which needs XphA for proper functioning (69). It has been suggested that the type II secretion system may have evolved from the type IV pilus assembly system. These two systems share striking similarity, and both use the prepilin peptidase PilD, which processes the pilin (including PilA) and pseudopilin (including XcpT) subunits. It is remarkable that XcpT can form a pilus when overproduced. However, the artificial elongation of the XcpT pseudopilus blocks the type II secretion process. This observation suggested that the physiological XcpT pseudopilus structure might instead consist of a brief sequence of elongation/retraction (powered by the traffic ATPase) within the periplasm. Such a "piston" mechanism leads to the expulsion of the exoproteins, transiently transported to the periplasm, into the extracellular medium via the secretin channel. Type IV pili are retractile appendages, and in that case extension of the structure far beyond the cell surface (through the secretin named PilQ) is physiologically relevant for adhesion and motility. Interestingly, some minor components of the type IV pilus, such as the PilY1 adhesin, are transported to the periplasm in a Sec-dependent manner and could have been brought to the cell surface thanks to the elongating type IV pilus. This process of transport of the PilY1 adhesin may thus be extremely similar to the transport of type II-dependent exoproteins. In the case of PilY1, the protein may have been carried by the type IV pilus all the way to the external medium and remained attached to the pilus. However, in the case of the type II-dependent exoproteins, the enzymes are expelled by the brief elongation of the type II pseudopilus and are released into the extracellular medium. This appears to be a remarkable example of evolution and adaptation of a unique system to different physiological functions.

Ana M. Misic (University of Wisconsin, Madison) gave a talk on type IV pilus retraction. As mentioned above, in *P. aeruginosa*, type IV pili are involved in biofilm formation, aggregation, cell adhesion, and twitching motility. Type IV pili are powered by the hexameric ATPases PilB and PilT, which are responsible for pilus elongation and retraction, respectively. The equilibrium between the two leads to the functional type IV pili and coordinated motility. The structure of PilT from *Aquifex aeolicus*, a hyperthermophile, was recently published (94). At the meeting, the structure of *P. aeruginosa* PilT was presented. PilT forms a hexamer in the crystal packing lattice, with three monomers per asymmetric unit. The PilT subunit is a bilobed protein, with an N-terminal PAS-like fold and a C-terminal domain consisting of a RecA fold, along with four far C-terminal helices. The PilT hexamer forms an interlocking torus, with the N-terminal domain of one subunit resting on the C-terminal domain of the adjacent subunit. The tripartite nucleotide binding site is found at the junction of three domains, the N- and C-terminal domains from one subunit and the C-terminal domain of the adjacent subunit. Despite great interest, the identity of PilT protein interaction partners remains elusive.

The type III secretion system translocates bacterial effector molecules directly into the eukaryotic host cell and contributes to virulence in many gram-negative pathogens. *P. aeruginosa* secretes up to four known effectors: ExoU, a potent phospholipase; ExoY, an adenylate cyclase; and ExoS and ExoT, highly homologous bifunctional proteins which possess an N-terminal GTPase-activating protein domain and a C-terminal ADP-ribosyl transferase domain (33). Joanne Engel (University of California, San Francisco) focused attention on the type III secreted protein ExoT. The GTPase-activating protein domain targets the Rho family GTPases Rho, Rac, and CDC42, while the ADP-ribosyl transferase domain modifies and interferes with the function of Crk, a scaffolding protein involved in the actin cytoskeleton (6). The host cell epithelium is a very effective defense barrier, and acute *P. aeruginosa* infections require pre-existing cell injury. Engel's lab has shown that both domains of ExoT inhibit wound repair through multiple mechanisms. In particular, both domains inhibit cell migration and cell proliferation, necessary components of wound healing. These studies further revealed a novel and unexpected role for Crk in cytokinesis, the final stage of cell division, in which the daughter cells separate. ExoT and Crk both localize to the midbody, the structure at which daughter cell membrane separation occurs (97). Finally, ExoT is necessary and sufficient to induce apoptosis (98). With ExoT as well as ExoU, a potent cytotoxin, or ExoS, *P. aeruginosa* has efficient and failsafe mechanisms to inhibit wound repair and propagate the epithelial injury, explaining at least in part its success as an opportunistic pathogen.

Alice Prince (Columbia University) compared various *P. aeruginosa* PAK strains for their ability to permeate airway epithelial cells and for their ability to cause invasion infection in a mouse model of pneumonia (102a). Wild-type PAK was able to transmigrate across monolayers of airway epithelial cells and cause increased permeability compared to mutants lacking either ExoS or ExoSTY, with the effect of the triple mutant being more severe than that of the single mutant. Similar effects were also seen in a mouse model of pneumonia. PAK also demonstrated dramatic effects on the tight junction protein occludin and on E-cadherin and actin. These effects were not obvious in the PAK strain lacking ExoSTY. These results suggest that the effect of these type III effectors is to facilitate the invasion of organisms across the mucosal barrier.

Expression of the *P. aeruginosa* type III secretion system is complex and subject to multiple levels of regulatory control. A number of environmental cues appear to induce type III secretory activity. These include Ca^{2+} limitation and host cell contact. The core regulator for this system is ExsA, a member of the AraC/XylS family of transcriptional activators. Additional proteins, including ExsC, -D, and -E, regulate ExsA-dependent transcriptional activity. Timothy Yahr (University of Iowa) presented a working model that suggests that ExsC, -D, and -E participate in a novel regulatory cascade that couples type III gene expression to the activity of the secretion channel. ExsD functions as an antiactivator by binding to and inhibiting ExsA-dependent transcription. ExsC functions as an antiantiactivator by binding to and inhibiting the negative reg-

ulatory activity of ExsD. Finally, ExsE inhibits ExsC. In the presence of limiting Ca^{2+} or host cell contact, ExsE is secreted from the bacterial cells or directly translocated into host cells (108). The corresponding reduction in the intracellular concentration of ExsE allows ExsC to bind to ExsD and thereby liberates ExsA to activate transcription. This elegant regulatory mechanism allows *P. aeruginosa* to rapidly respond to and intoxicate host cells using the type III secretion mechanism.

P. syringae uses type III secretion to cause disease. Without this secretion system, plants resist infection. Jeff Chang (Oregon State University) hypothesized that type III secretion effectors inhibit basal host defense. He noted that individual races of *P. syringae* are polymorphic in their collection of type III effectors. In order to identify these effectors, a high-throughput screen was developed and used to identify type III effectors from 16 different strains of *P. syringae*. The screen was based on induction by HrpL and delivery directly into host cells. Thus far, Chang has identified 300 to 500 putative effectors from this screen.

Marshall L. Hayes (Cornell University) investigated the factors influencing type III secretion in *P. syringae* pv. tomato DC3000. The factor myoinositol, a precursor in plant cell wall biosynthesis, was added to *P. syringae* strains carrying luciferase-based type III secretion system transcriptional reporters. His results suggest that this molecule may be active during early stages of bacterial sensing of the host environment, where it then responds by regulating type III secretion system genes.

Finally, Michael L. Vasil (University of Colorado Health Sciences Center) presented data on a particular class of extracellular phospholipases C of *P. aeruginosa*. These enzymes are released into the extracellular environment by the twin arginine translocase and type II secretion systems. One of these, PlcH, is highly cytotoxic for human vascular endothelial cells and kills them through apoptosis. However, it is relatively weakly toxic to other kinds of eukaryotic cells (e.g., epithelial cells and fibroblasts). PlcH may also have a role in sepsis by allowing *P. aeruginosa* to break through the endothelial cells to the vascular system and inhibit wound healing by specifically killing proliferating endothelial cells that are required for this process. These properties of PlcH may also play a role in the establishing a perivascular location for *P. aeruginosa* during sepsis. PlcH appears to bind specific endothelial integrin receptors through an Arg-Gly-Asp (RGD) motif. Integrin binding and toxicity to human vascular endothelial cells can be inhibited by RGD peptides, which appear to compete with PlcH for the specific integrin receptor for PlcH. In addition to its role as a virulence factor, through its inhibition of angiogenesis and wound healing, this enzyme could possibly be used to treat diseases in which angiogenesis contributes to the pathogenesis of noninfectious diseases, such as tumor growth and ocular macular degeneration.

NEXT MEETING

The next *Pseudomonas* meeting will take place 20 to 24 August 2009, in Hanover, Germany, and will be hosted by Burkhard Tümmler.

ACKNOWLEDGMENTS

We thank the scientific program organizers, especially Caroline S. Harwood (University of Washington), for hosting such an outstanding meeting. We also thank the American Society for Microbiology staff, including Lisa Nalker, Traci Williams, and Latonya Nichols, for making this meeting a great success. We appreciate the conference speakers for editing their own sections of this review for accuracy and content.

We also thank the Cystic Fibrosis Foundation, Novartis, Gilead Sciences, and Merck Research Laboratories for financial support of this meeting.

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