MEETING REVIEW

Biofilms 2003: Emerging Themes and Challenges in Studies of Surface-Associated Microbial Life

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For over a century microbiologists have studied liquid cultures of bacteria. In fact, a common criterion for choosing a microorganism to study has been its ability to grow in a suspended, homogeneous culture format, thereby simplifying examination of microbial physiology and genetics. Although these studies have been tremendously informative, they neglect the observation that many bacteria in the natural environment grow aggregated with each other, with solid surfaces, and at gas-liquid interfaces. There is a growing appreciation that, although clearly worthwhile, studies of standard planktonic cultures provide us with a biased view of microbial life.

The study of microbial biofilms has received significant attention and achieved significant popularity in the last decade. As the numbers of laboratories and scientists interested in biofilms have rapidly increased, the field has suffered some growing pains. Anyone wishing to conduct biofilm research or to compare their results with those of other laboratories faces the distinct problem of the limited number of standardized systems or protocols for studying biofilms. Another challenging aspect of the field is its multidisciplinary nature. Biofilms are important in environmental, industrial, and clinical contexts (16, 19, 99). The study of biofilm communities benefits from the efforts of investigators from many different disciplines, including environmental and clinical biologists, surface chemists, engineers, and mathematical modelers, who bring their unique questions, perspectives, and technologies to bear on this phenomenon. Unfortunately, it's difficult to keep abreast of the scientific literature in one's own field, let alone others. The rapid growth of biofilm research and the need to bring together people from different disciplines interested in biofilms led the American Society for Microbiology (ASM) to sponsor Biofilms 2003, which was held in Victoria, Canada, on 1 to 6 November 2003. This was the third such meeting in a series, with the previous two being held in Snowbird, Utah, in 1996 and Big Sky, Mont., in 2000. There were 638 participants including 260 international scientists representing 36 countries and 112 graduate and undergraduate students. The meeting was divided into six sessions spread over 4 days. One day was set aside for biofilm workshops and demonstrations. There was a mix of invited speakers and those selected from submitted

abstracts. Finally, evening breakout sessions were held on four of the nights. These sessions were organized to provoke a round-table discussion of key research topics. Most conferees agreed that although the days were long, the scientific discourse the meeting generated and the information shared were outstanding.

Three keynote lectures were given on different evenings of the conference. These talks each captured critical aspects of the field and helped to set the tone of the meeting. The first was given by J. William Costerton, Director of the Center for Biofilm Engineering in Bozeman, Mont. Costerton reminded us how far we have come in the field and emphasized the point that we continually tend to underestimate the ability of bacteria to coordinate behaviors and processes as a community. David Stahl of the University of Washington gave the second keynote address. He provided us with examples of how studying pure cultures of organisms in the laboratory can mislead us and fail to explain observations of their behavior in the context of environmental communities. In the final keynote address, Søren Molin of the Danish Technical University pointed out that biofilm microbiology is a field that relies heavily on microscopic observation. He described the problems of interpreting such data and cautioned that alternative explanations are possible for what may appear to be a straightforward result. With this as the underpinning theme of his talk, he then proceeded to challenge several points of emerging biofilm dogma that are based primarily on microscopic data. The meeting at Victoria also marked a special occasion to recognize the career of J. William Costerton at an opening-night reception. He took the lead role in organizing the first three ASM-sponsored biofilm conferences and has worked tirelessly to promote and to spread the biofilm concept.

A distinct impression taken from this meeting was the great number of laboratories doing high-quality research. Much of the work presented at the previous meeting in 2000 was fairly observational and had a qualitative feel to it. In Victoria, it was clear that many laboratories were conducting reductionist research and asking sophisticated questions. Another impression was how advances in imaging technology have transformed the field. At the 2000 meeting, only a few laboratories were capable of sophisticated microscopy, while at the Victoria meeting reports of confocal and time-lapse microscopy were commonplace. The great breadth of the meeting was reflected by the oral platform, which was organized into six sessions. These sessions were (i) Biofilm Structure/Function and Physiology,

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(ii) Developmental Patterns in Biofilms, (iii) Biofilms in Natural and Industrial systems, (iv) Cross Kingdom Interactions, (v) Pathogenesis, and (vi) The Biofilm Phenotype. Rather than providing a summary of every talk, this review intends to capture emerging themes and report key interesting new findings presented at the meeting. The following topics were the focus of attention and discussion.

ANTIMICROBIAL RESISTANCE

A general characteristic of biofilm communities is that they tend to be significantly more resistant to antibiotics and antimicrobial stressors, including those represented by host-defense responses, than planktonic bacteria of the same species (35, 65, 97, 98). The general consensus in the field is that no one single property of a biofilm can universally explain this heightened resistance—it is probably the manifestation of a number of factors. Biofilm properties thought to contribute to antimicrobial resistance include those that affect the penetration of the antimicrobial, the metabolic activity of biofilm cells, and phenotypic variability within the biofilm.

Cells within a biofilm are usually enmeshed within a matrix of extracellular polymeric substances (EPS), primarily produced by the microorganisms themselves. The EPS matrix may affect the penetration of an externally applied antimicrobial stress to cells buried in the depths of a biofilm. The general consensus is that certain antibiotics such as fluoroquinolones penetrate *Pseudomonas aeruginosa* biofilms readily while other antibiotics, such as aminoglycosides, penetrate more slowly since they bind to extracellular polymers such as alginate (38, 76, 100, 109). Reduced penetration has been suggested to contribute to biofilm cells evading components of the host immune response (16, 55). However, Jeff Leid reported in Victoria that human leukocytes could in fact penetrate *P. aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* biofilms. He measured the response of these leukocytes and found that they produce cytokines indicative of a Th1-type response. Leid also reported that genetic factors important for biofilm antibiotic resistance (such as *ndvB* of *P. aeruginosa*, mentioned below) also affect susceptibility to human leukocytes. Michael Givskov used confocal microscopy to study the interaction of human polymorphonuclear leukocytes with *P. aeruginosa* biofilms. He reported that biofilms formed by a quorum-sensing *lasR rhlR* double mutant are more susceptible to polymorphonuclear leukocyte-mediated killing than wild-type biofilms.

Cells within a biofilm are subject to nutrient gradients, which usually result in metabolically active cells, which have access to nutrients in the overlying liquid at the periphery of the biofilm, and metabolically inactive cells within the interior. Most antibiotics target actively growing cells, so cells in the interior would be protected from antibiotic killing (9, 94). Phil Stewart reported a novel approach to assess metabolic heterogeneity in a *P. aeruginosa* biofilm community. Using the *tac* promoter fused to a stable version of the green fluorescent protein (GFP), he used de novo protein synthesis as an assay for metabolic activity. Using biofilms either grown on a filter placed on solid growth medium or in glass capillary tubes, he monitored inducible fluorescence in the community and found that fluorescence localized to the periphery of the biofilm. This

zone of fluorescence corresponded to oxygen gradients measured with microelectrodes. Oxygen, besides being required for GFP folding, has been shown to be a limiting substrate for biofilm growth for this organism (117). This strategy has been used to show that zones of metabolic activity correspond to zones of antimicrobial sensitivity.

Biofilm bacteria exhibit specific physiologies and patterns of protein and gene expression when associated with a surface, compared to planktonic cells (86, 113). This has been loosely dubbed the “biofilm phenotype.” The phenotypic differences between biofilm and free-swimming cells has been proposed to partially explain the heightened resistance of biofilm cells. Several interesting new findings presented at the meeting shed light on the impact of the biofilm phenotype on antimicrobial resistance. George O’Toole’s group identified a locus on the *P. aeruginosa* PA14 chromosome involved in the resistance of biofilm communities to the aminoglycosides tobramycin and gentamicin and the fluoroquinolone ciprofloxacin (68). A mutation in the *ndvB* gene rendered *P. aeruginosa* biofilms over an order of magnitude more sensitive to these antibiotics compared to the wild-type strain, while not affecting planktonic populations. They went on to demonstrate that *ndvB* is expressed only in biofilm communities and based on sequence similarity and initial experimental evidence, predicted that this gene may be involved in directing the synthesis of periplasmic cyclic glucans. O’Toole proposed that these cyclic glucans may bind and sequester these antibiotics in a biofilm community.

Numerous laboratories have made the observation that after a majority of a biofilm population has been killed by an antimicrobial, a very small percentage of the population remains viable despite prolonged exposure to the antibiotic or increased dosage. These cells are called “persisters” and confer no heritable resistance to progeny once the selective pressure is removed (27, 54, 74, 88, 94, 114). The exact physiology behind the persister phenotype is unknown; however, the *hip* genes (for high level of persistence) have been suggested to control the frequency of the persister phenotype in *Escherichia coli*. At the meeting, Peter Gilbert discussed his group’s work investigating the persister phenotype. Using a constitutive promoter fused to GFP in combination with fluorescence-activated cell sorting, Gilbert’s group was able to show that a homogenous population of liquid culture-grown cells contained a bell curve distribution of fluorescence intensities. The end of the curve representing cells that were less bright than the rest of the population contained an unusually high level of cells with the persister phenotype (101). This led to the hypothesis that this phenotype is linked to a metabolically less active subpopulation that is always present within a given population.

Mark Schinabeck described antibiotic resistance in the fungus *Candida albicans*. He described three stages of biofilm development for this organism. In the earliest stage of biofilm development, *C. albicans*, like planktonic populations, relied on the activity of two efflux pumps, the Cdr and Mdr systems, for resistance to the antifungal compound fluconazole. At the two later stages of biofilm development, these pumps were not necessary for resistance. This led to an analysis of biofilm cells isolated from later stages of development. These “aged” biofilm cells were shown to have significant changes in the sterol composition of their membranes, suggesting that differential

regulation of membrane sterol content is a mechanism for increased resistance in older biofilms.

THE EPS MATRIX

One of the most distinctive features of biofilms when compared to planktonic populations is that the cells are embedded in EPS. We know little about this matrix (15, 103). This is in large part due to its dynamic nature. Characterizing the composition of the EPS matrix is a daunting proposition. The composition of EPS varies depending upon the organisms present and environmental conditions. Presumably, under the proper circumstances, cells can and will attempt to influence their surrounding chemical and physical environment by secreting specific biological macromolecules. However, this environment can also harbor components of abiotic origin as well as matrix nonspecific biological molecules derived from the lysis of cells. The challenge is determining when, why, and how a community regulates EPS composition and the ultimate functional consequences of this behavior.

Several talks and posters at the meeting provided new insight concerning this matrix. For many years alginate, a polysaccharide polymer consisting of mannuronic and guluronic acids, was thought to be the major EPS polysaccharide of nonmucoid strains of *P. aeruginosa*. Recent results have indicated that this is not the case, leaving the question what, if any, polysaccharide is an important component of nonmucoid EPS (31, 43, 78, 116). Three groups converged on this question independently. E. P. Greenberg, Roberto Kolter, and D. J. Wozniak and M. R. Parsek independently identified a locus on the PAO1 chromosome, now designated the *psl* locus, that harbors a cluster of genes (PA2231 to PA2245) showing homology to exopolysaccharide biosynthetic genes. Mutations in the *psl* locus gave *P. aeruginosa* a biofilm attachment-deficient phenotype. Kolter suggested that this cluster of genes might encode functions for production of a polysaccharide rich in mannose and glucose. Interestingly, in *P. aeruginosa* PA14, a separate locus designated *pel* was identified that is involved in producing a polysaccharide involved in biofilm development for this strain (31). This locus was identified by screening microtiter plate-grown biofilms defective in forming a pellicle at the liquid-air interface. These studies suggest that polysaccharides distinct from the well-studied alginate play important roles in *P. aeruginosa* biofilms. Similarly, Clay Fuqua reported that *Agrobacterium tumefaciens* mutants unable to synthesize the well-characterized exopolysaccharide succinoglycan are unaffected for biofilm formation and these mutant biofilms contain one or more different polysaccharides.

Susanne von Bodman presented a poster (S. B. von Bodman, M. Koutsoudis, C. Herrera, and T. D. Minouge, Biofilms 2003, abstr. 184, 2003) describing the relationship between acyl-homoserine lactone (HSL)-based quorum sensing and production of the extracellular polysaccharide stewartan by *Pantoea stewartii*, a bacterial pathogen of sweet corn and maize. The quorum-sensing regulators, EsaI and EsaR, were shown to control stewartan synthesis (107). Quorum-sensing mutants appeared to be impaired in biofilm formation and development, although these mutants show increased initial attachment to surfaces. Luanne Hall-Stoodley and H. Lappin-Scott characterized the biofilm matrix of *Mycobacterium fortuitum*

FIG. 1. Three-dimensional reconstructions of *P. aeruginosa* biofilms formed in a flow cell reactor after an initial 1:1 inoculation of the system with yellow fluorescent protein-tagged wild-type strain PAO1 and a cyan fluorescent protein-tagged *pilA* mutant defective in type IV-mediated twitching motility. The stalks of the biofilm mushroom structures were formed by clonal growth of the nonmotile *pilA* mutant. The caps of biofilm mushroom structures were formed by the wild-type strain, which migrated up the stalks of the *pilA* mutant. Magnification, $\times 400$. (Image courtesy of Mikkel Klausen and Tim Tolker-Nielsen, Danish Technical University).

(39) with lectin and lipophilic fluorescent stains. *M. fortuitum* stained with the mannopyranose- and glucopyranose-specific lectin concanavalin A. The matrix also stained with lectins specific for *N*-acetylgalactosamine. When lectin and lipophilic stains were used in conjunction with nucleic acid stains, biofilms showed regions of lipophilic or carbohydrate staining without concomitant nucleic acid staining, suggesting an extracellular matrix. This was particularly pronounced with the lipophilic staining patterns, raising the question of whether the *M. fortuitum* matrix is comprised of excreted lipids or dead cells that have lost nucleic acid.

DYNAMICS OF BIOFILM FORMATION AND MATURATION

Tim Tolker-Nielsen presented some fascinating data describing the multicellular behavior involved in the formation of mushroom structures found under certain growth conditions for *P. aeruginosa* biofilms (56). Using surfaces seeded with mixed populations of wild-type bacteria fluorescently tagged with either the cyan or the yellow variant of GFP, his group showed that the stalks of mushrooms are formed by clonal growth of a distinct population, whereas the caps of the mushrooms are formed by a distinct motile subpopulation that migrates up onto the stalk. Time-lapse microscopy and experiments with twitching motility mutants supported this model for the formation of mushrooms (Fig. 1).

Staffan Kjelleberg presented evidence that a PF1-like filamentous phage present on the PAO1 chromosome is induced in older *P. aeruginosa* biofilms, killing cells in the interior of the biofilm (111). Kjelleberg's group has identified other organisms, *Serratia marcescens*, *Vibrio cholerae*, and *Pseudoalteromonas tunicata*, that may also induce killing within older biofilms. Kjelleberg likened this to a form of apoptosis and drew a

parallel between *P. aeruginosa* biofilm formation and *Myxococcus xanthus* fruiting-body formation. He hypothesized that this may be a mechanism for generating new nutrients in starved biofilms. Allain Filloux presented his group's work on the relationship of the three clusters of genes encoding the chaperone-usher fimbrial assembly pathways to biofilm formation in *P. aeruginosa* (105). He showed that the *cupA* cluster is required for biofilm formation on abiotic surfaces, whereas the *cupB* cluster is important for adherence to bronchial epithelial cells. He also identified a regulatory protein, MvaT, that controls expression of the *cup* clusters as well as other adhesins.

Quorum sensing is a term used to describe cell-to-cell signaling systems. Most gram-negative organisms utilize an acyl-HSL-based signaling system, while most gram-positive organisms use secreted peptides. Previous work has implicated quorum sensing in biofilm formation in a number of organisms. The relationship between quorum sensing and biofilm formation was a subject that dominated much of the 2000 meeting; however, there was considerably less material presented at Victoria. Acyl-HSL-based quorum sensing in *P. aeruginosa* has been previously linked to biofilm formation (20, 47, 61, 67). Michael Givskov's group characterized a number of quorum-sensing inhibitors (QSIs) and their effect on *P. aeruginosa* biofilm formation and function. Perhaps the best-characterized QSIs are the halogenated furanones, first isolated from the seaweed *Delisea pulchra* (37, 42, 44, 69). The Givskov group used a DNA microarray approach to show that addition of this furanone to *P. aeruginosa* cultures inhibited activation of quorum-sensing-regulated genes (44). He went on to show that treatment of *P. aeruginosa* biofilms with this inhibitor rendered them more susceptible to a variety of antimicrobial compounds. Thomas Rassmussen from the Givskov group presented a separate study, in which they used a screen to identify QSIs from natural sources. Several candidate compounds were isolated, with one rather potent inhibitor isolated from garlic. Some of these QSIs also inhibited quorum sensing in the lungs of mice.

Biofilm formation in gram-positive organisms was also well represented in Victoria. Naomi Balaban and colleagues used a linear heptapeptide called RIP (RNAIII-inhibiting peptide; RNAIII is the quorum-sensing regulatory effector identified in *S. aureus*) to target the peptide-based quorum-sensing systems of *S. aureus* and *S. epidermidis* (5, 34). This work utilized an animal model of infection, specifically grafts inserted subcutaneously in rats. Grafts pretreated with RIP showed reduced bacterial loads after extended exposure. Balaban suggested that RIP may be an effective therapeutic agent to target *S. aureus* and *S. epidermidis* biofilm infections. Jeremy Yarwood analyzed the contribution of the *agr* quorum-sensing system of *S. aureus* in biofilm formation. Yarwood found that an *agrD* mutant formed biofilms with less biomass in a spinning disk reactor, while the mutation had no effect on biofilm formation in a flow cell. He used a transcriptional fusion of the P3 promoter (controls expression of the RNAIII gene) to GFP and showed that RNAIII expression occurred in successive waves throughout a biofilm followed by waves of detachment, suggesting that *agr* expression may induce detachment.

Beth Lazazzera presented work describing genes involved in biofilm formation in *Bacillus subtilis* (40, 95). She described the regulatory circuitry which determines if *B. subtilis* will form a

spore or a biofilm. She demonstrated that mutations in Spo0A caused deficiencies in *B. subtilis* biofilm formation. Lazazzera went on to hypothesize that sporulation is not essential for biofilm formation. Further experiments led her to propose that low Spo0A levels determine that *B. subtilis* will form a biofilm, while high levels determines that it will form spores.

PHENOTYPIC DIVERSIFICATION IN BIOFILM COMMUNITIES

A number of presentations throughout the meeting had the common thread that significant phenotypic diversification occurs within biofilm communities. Presumably, this diversification reflects adaptation to microenvironments found within a biofilm. Biofilm communities experience a wide range of gradients, which result in a landscape of microniches and the accompanying selective pressures. Evidence was presented that diversification can produce variants with biofilm-specific phenotypes. Mary Jo Kirisits and Pradeep Singh reported independently that growth of *P. aeruginosa* as a biofilm produced a number of colony morphology variants when older biofilms were resuspended and plated on solid growth medium. One of these variants, called the "wrinkly" or "sticky" variant, formed small rough colonies on solid growth medium and displayed a hyper-biofilm-forming phenotype on abiotic surfaces. Similar phenotypes have been reported in the literature for a number of other species including *Salmonella enterica* serovar Typhimurium and *V. cholerae* (118, 120). Biofilms formed by the sticky variant showed heightened resistance to the antibiotic tobramycin and the biocide bleach compared to biofilms formed by the wild-type parental strain. Similar observations have been reported in the literature concerning this *P. aeruginosa* phenotype (21, 23, 41). Singh also reported the isolation of "small" colony variants, with a significantly smaller diameter than that of the wild-type parent when grown on solid medium. The small-colony variant displayed a hyperdispersion phenotype and will be discussed in the following section. Kirisits concluded by showing that 20 randomly selected isolates derived from an aged biofilm with wild-type colony morphologies on solid growth medium displayed a wide range of swimming and twitching motility activity. These were heritable changes, suggesting that colony morphology variants represented a small fraction of the diversity present in the population.

Eliana Drenkard reported the isolation of rough, small-colony variants (RSCVs) from *P. aeruginosa* PA14 (23). These variants were not isolated directly from biofilms but from planktonic cultures subjected to antibiotic selection. RSCVs displayed a hyper-biofilm-forming phenotype on abiotic surfaces and increased antibiotic resistance. A gene encoding a putative transcriptional regulator, *pvrR*, was identified that controlled the switch from an RSCV to a wild-type phenotype. Drenkard also reported that PvrR is involved in the regulation of biofilm formation and resistance to antibiotics. This research provided a link between antibiotic resistance, biofilm formation, and the RSCV-like variants found in cystic fibrosis patients. Andrew J. Spiers (A. J. Spiers, S. Gehrig, J. Bohannon, Z. Robinson, and P. Rainey, Biofilms 2003, abstr. 3, 2003) described a colony morphology variant of *Pseudomonas fluorescens* dubbed the "wrinkly spreader" (93). This variant resulted from mutations in the *wss* operon, which shows homol-

ogy to genes involved in the acetylation of the exopolysaccharide alginate. This variant produces an acetylated form of cellulose that allows the variant to colonize a specific niche—the air-liquid interface of standing liquid cultures. While the concept and impact of diversification are most likely important, much work remains to be done to determine the mechanisms by which diversity is generated, the identification of phenotypes with specific niche specialties, and the impact of diversification on the pathogenic or ecological potential of a community.

There were also data presented at the meeting showing that plasmids can have a significant impact on the biofilm-forming properties of a strain, with intriguing implications regarding horizontal gene transfer. Jean Marc Ghigo expanded upon his earlier published observation that conjugal plasmids can promote biofilm formation. He reported the presence of an antigen 43 homolog on the F plasmid of *E. coli*. Antigen 43 is an aggregation factor that promotes biofilm formation. Enhanced biofilm formation by a strain of *E. coli* MG1655 harboring the F plasmid was shown to be linked to F-pilus production (6, 33). On the other hand, Lori Burrows reported that in certain instances plasmid-encoded functions impair biofilm formation. The TEM-1 gene, used as a marker for ampicillin resistance in *E. coli*, had a negative effect on biofilm formation. Inactivation of this gene, specifically the encoded beta-lactamase activity, restored normal biofilm formation. This observation extended to other commonly used ampicillin resistance genes. Burrows hypothesized that encoded beta-lactamases bind to peptidoglycan, competing with penicillin binding proteins, which have been shown to be important for biofilm formation. Her talk provided a sobering word of caution regarding evaluating the biofilm phenotype of an organism after addition of new genetic capabilities, no matter how innocuous they may seem.

DISPERSION OR DISSOLUTION

There is building evidence that certain species of bacteria can actively leave a biofilm in a process that has been termed dispersion or dissolution. This is presumably achieved by coordinating the breakdown of the surrounding extracellular matrix through the action of secreted (e.g., polysaccharide lyases) or cell surface-associated enzymes, with the activation of motility functions (Fig. 2). This activity is thought to represent a final step in biofilm development, in which biofilm cells return to the planktonic state. Karin Sauer described dispersion in *P. aeruginosa*, a process she could reproducibly induce in biofilms by changing the culture conditions (e.g., a rapid decrease in pH). Dispersion was accompanied by induction of genes involved in flagellar swimming motility and repression of the gene, *pilA*, encoding the type IV pilus structural subunit. She also demonstrated that the transition from a biofilm to a planktonic state was accompanied by large changes in protein phosphorylation patterns as assayed by two-dimensional gel electrophoresis. David Davies reported the isolation of a factor from the spent culture fluid of *P. aeruginosa* that upon addition could induce dispersion in a biofilm. Although the identity of this factor is unknown, it is heat stable and smaller than 5 kDa in size. Paul Stoodley described a similar phenomenon he termed “seeding dispersal,” in which cells leave a biofilm microcolony at a particular minimum size threshold of 100 μm in

FIG. 2. Detachment process as observed in wild-type *P. aeruginosa* biofilms. In older mature biofilms, central cavities develop that become filled with swimming bacteria. Dispersion occurs when the cavities rupture and motile bacteria are released. Represented is a PAO1 biofilm cultured in a flow cell reactor. Cells are constitutively expressing GFP. Magnification, $\times 630$. (Image courtesy of Pradeep Singh, University of Iowa).

diameter. The mechanism appears to involve activation of swimming motility and, interestingly, does not occur with tested mucoid backgrounds of *P. aeruginosa*. Pradeep Singh described the isolation of a small-colony variant of *P. aeruginosa* that showed an enhanced dispersion/dissolution phenotype. This variant was shown to overproduce rhamnolipids. Rhamnolipid biosynthetic mutations generated in the variant eliminated the enhanced dispersion phenotype. Furthermore, addition of exogenous rhamnolipid could induce premature dispersion in wild-type *P. aeruginosa* biofilms.

Jeffrey Kaplan described dispersion in a nonmotile, gram-negative oral pathogen, *Actinobacillus actinomycetemcomitans*. This organism aggressively forms biofilms on abiotic surfaces (52, 53). The *A. actinomycetemcomitans* dispersion mechanism appears to involve ejection of single cells or small clusters of cells from the interior of the biofilm into the overlying liquid. Transposon mutagenesis identified dispersion-defective mutants. Functions required for dispersion were related to lipopolysaccharide O-side-chain biosynthesis; PtsI, a regulator of sugar uptake and catabolite repression; and DspB, a beta-hexosaminidase. Alfred Spormann described genetic elements involved in biofilm detachment for the Fe- and Mn-reducing bacterium *Shewanella oneidensis* MR1. Detachment events were controlled by oxygen tension in a flow cell reactor system. Spormann's group identified an FNR-homolog, EtrA, and a two-component response regulator, ArcA, that are involved in sensing oxygen levels and controlling the detachment process.

Future work will probably uncover a variety of dispersion/dissolution mechanisms utilized by different species. These

mechanisms represent an attractive target for antibiofilm therapy.

THE BIOFILM PHENOTYPE

One current area of intense focus in biofilm research has been determining the “biofilm phenotype” of different organisms. The biofilm phenotype is loosely defined as the patterns of protein and gene expression associated with biofilm cultures in comparison to those associated with planktonic culture. The general hope is that such studies may identify candidate genes or proteins that may explain some of the antibiotic resistance phenomena associated with biofilms. Several studies have been reported in the literature, and even the most conservative estimate reports significant changes in expression patterns (85–87, 113). Several groups presented findings describing global genomic or proteomic studies of biofilm protein and transcriptional profiles of a variety of interesting organisms other than some of the traditional model gram-negative proteobacteria. Mark Shirtliff reported his proteomic analysis of planktonic and biofilm *S. aureus*, noting that approximately 20% of the proteome was differentially regulated when comparing liquid and biofilm cultures. Specific proteins identified included enzymes involved in central metabolism that were upregulated in older biofilms and a membrane-bound potassium transporter. Microarray experiments validated the trends seen with proteomics. Shirtliff voiced the dilemma facing anyone conducting such a study—how does one sift through and evaluate all the data? Phil Marsh evaluated changes in gene expression associated with intracellular growth inside macrophages in the pulmonary pathogen *Mycobacterium tuberculosis*. A GFP-based approach was employed using differential fluorescence induction as a readout in conjunction with a cDNA microarray analysis. Using differential fluorescence and a promoter trap strategy, he sorted cells by fluorescence-activated cell sorting. This study identified genes upregulated in macrophages involved in cell wall biosynthesis as well as a number of genes with unknown function. Hillary Lappin-Scott reported her DNA microarray-based analysis of *S. enterica* serovar Typhimurium biofilms. She found upregulation of the thin aggregative fimbriae called curli and cell division-associated genes. One of the more interesting results was that they observed downregulation of all but one of the salmonella pathogenicity islands, perhaps suggesting that downregulation of genes involved in acute, invasive infection is coordinated with the onset of chronic infection. She also observed that swimming motility functions were both up- and downregulated in biofilms. Jean Marc Ghigo presented work determining the transcriptional profiles of mature *E. coli* K-12 biofilms. He showed that 10% of the *E. coli* genome in biofilms is significantly differentially expressed compared to that in logarithmically growing planktonic cells. This work provided evidence that the expression of stress envelope response genes, such as the *psp* operon or elements of the *cpx* and *rpoE* pathways, is a general feature of *E. coli* mature biofilms (6).

INTERSPECIES INTERACTIONS

Outside of certain types of infections or symbioses, most biofilms consist of multiple species of both eukaryotic and

prokaryotic organisms. There are obvious consequences to different organisms in a biofilm being present in high density and close proximity. The potential for interspecies communication, competition, and cooperation is high. How different species perceive and respond to one another is a key feature of any multispecies system. Although this fact is widely appreciated, most of the biofilm research being conducted in the field today involves pure-culture systems. This is not surprising—trying to study the complexity present in simple, pure-culture biofilms is daunting enough. Microbiologists studying complex environmental systems have developed wonderful, sophisticated tools to determine what organisms are present, where they are, and their metabolic disposition. However, the tractability of natural, complex communities is limited. Important questions such as the physiological response of one population to another and how this impacts community structure and function are usually difficult to answer. Researchers have taken an alternative route by developing closed, artificial multispecies systems, where the composition of the community and environmental parameters can be carefully controlled. The downside to this approach is that the system is artificial and may not accurately represent the complex community being modeled. The advantages are that the researcher can ask basic reductionist questions and take advantage of technologies such as genomics/DNA microarrays and proteomics.

The meeting had a number of excellent presentations on the subject of interspecies interactions. Morten Hentzer described his model system for studying *P. aeruginosa* and *Burkholderia cepacia* interactions in the context of cystic fibrosis lung infections, where they can be found together. Both bacteria utilize acyl-HSL-based quorum sensing to regulate virulence factors (28, 32, 91, 92). Hentzer conducted a DNA microarray analysis of *P. aeruginosa* grown alone and in coculture with *B. cepacia*, finding that ~50 genes were differentially expressed in coculture. Interestingly, no quorum sensing-regulated genes appeared to be differentially regulated, although *P. aeruginosa* in pure culture was able to respond to exogenous addition of the *B. cepacia* purified signal molecule, octanoyl-HSL.

Several studies of eukaryote-prokaryote interactions were also presented. Anne Dunn from Jo Handelsman's laboratory described the interaction of the soil microbe *Bacillus cereus* and tomato plants (25, 26). She described *B. cereus* genes induced by the plant host, studied by using a fluorescence-based promoter trap strategy. Two promising genes were identified, *tspX*, a protease homolog, and *lipA*, a putative chaperone. Mutations in *lipA* rendered *B. cereus* competition defective in colonizing tomato seeds. Ann Hirsch presented work on the rhizobium-legume symbioses, using alfalfa and either *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, or *Rhizobium leguminosarum* as a model system. She described the importance of lectins in plant-symbiont interactions (106). Rhizobial strains that normally do not nodulate alfalfa would establish nodules on roots of transgenic alfalfa expressing different lectins. She then described a biofilm screen of mutants that indicated *S. meliloti* used many of the same factors to colonize abiotic surfaces and roots. Work presented from the Fuqua lab studying *A. tumefaciens* biofilms reported identification of an FNR-type regulator called SinR that controls biofilm maturation on model surfaces and on *Arabidopsis* tis-

sues and functions as a component of an oxygen limitation response pathway.

Ian Joint reported on the role that acyl-HSL signaling plays in zoospore settlement by the green alga *Enteromorpha* (49, 81). His previous published work demonstrated that older biofilms of *Vibrio anguillarum* are able to promote zoospore settlement through acyl-HSL signaling. Investigation of the algal partner indicated that acyl-HSLs elicit a calcium influx event. Joint went on to propose that plasma membrane ion channels might play a role in this signaling event in *Enteromorpha*. Anthony Smith reported his latest findings in another prokaryote-simple eukaryote interaction, *P. aeruginosa* and the protozoan *Acanthamoeba polyphaga*. Smith demonstrated that *P. aeruginosa* survives the intracellular environment of *Acanthamoeba*. Several different mutants were tested, and although type III secretion mutants had no phenotype in uptake or intracellular survival, *rpoS* mutants showed enhanced uptake and reduced intracellular survival. This led Smith to propose that *rpoS* plays an important role in allowing *P. aeruginosa* to survive predation by this protozoan.

BIOFILMS AND CHRONIC INFECTION

Although defining what constitutes a biofilm infection remains to be resolved, most clinicians agree that biofilms are responsible for a variety of chronic bacterial infections (16, 80). Bacteria tend to stick to and colonize foreign bodies or dead tissue in the human body. Many of these infections are caused by opportunistic pathogens that are also human commensals (16, 22). Clinical biofilms have received a great deal of attention in the last 10 years. Much recent research has focused on identifying genetic factors that contribute to biofilm formation and assessing the relative antimicrobial susceptibility of different key mutants that exhibit a biofilm phenotype. One biofilm disease in particular that has received much attention is cystic fibrosis (CF) airway infections. The evidence that biofilms play a role in CF pathogenesis is steadily building (14, 62, 72, 89). CF lung infections are chronic and extremely difficult to treat with antibiotics. Microscopic evidence shows striking images of bacterial aggregates present both in the sputum of patients and colonizing the surface lung airways, supporting the contention that CF infections adopt a biofilm conformation. *P. aeruginosa* present in CF sputum also produces acyl-HSL signals in ratios similar to those produced by laboratory biofilms. Several presentations at Biofilms 2003 focused on CF airway infections. Gerd Doering presented his group's work on the "anaerobic hypothesis" (8, 115, 119). This hypothesis is that oxygen tension in CF airways is very low and that *P. aeruginosa* relies on anaerobic metabolism to grow as a biofilm in CF airways. According to the hypothesis, *P. aeruginosa* grows on mucous plugs in the lung and according to microsensor measurements, oxygen gradients in the plugs are very steep, with much of the system being anaerobic. Using antibodies specific to the exopolysaccharide alginate, his group showed that alginate production is induced in this environment. Claus Moser reported on the effect of the macrolide azithromycin on the immune system in a murine model of chronic infection. According to Moser, the balance of the Th1/Th2 immune responses is key to features of chronic infection (73). Although azithromycin is ineffective against *P. aeruginosa* as an antibiotic, in the murine

model it stimulated an increase in the number of and activation state of CD4⁺ cells and a decrease in interleukin-4. A similar analysis was conducted with azithromycin-treated CF patients, where a decrease in the interleukin-4/gamma interferon ratio was observed. Collectively, these data suggest that azithromycin asserts its effect through modulation of the immune response in CF.

Another area of marked growth in the field since the meeting in 2000 was the significant amount of new research on pathogenic organisms other than *P. aeruginosa*. Greg Anderson of Scott Hultgren's laboratory, presented his work on the role of biofilms in urinary tract infections by uropathogenic *E. coli* (2, 50). He presented microscopic data suggesting that *E. coli* invades urinary epithelium and establishes intracellular "pods" or biofilms that help *E. coli* evade the host immune response. *E. coli* within these pods is enmeshed in an EPS network and produces type 1 pili and antigen 43, factors known to participate in biofilm development for this bacterium. Another organism that has been implicated in biofilm infections is *Enterococcus faecalis*, which can cause endocarditis and post-operative infections. A surface protein, Esp, had been previously identified as important for biofilm formation for this organism (104). C. J. Kristich of Gary Dunny's laboratory reported that many Esp-deficient strains have been isolated from patients suffering from *E. faecalis* infections, suggesting that Esp may not be required for biofilm formation by all strains. Kristich presented data on a plasmid-free strain, *E. faecalis* OG1RF, that is Esp deficient and found that indeed this organism is capable of forming a biofilm in vitro (58). Biofilm formation was also highly dependent upon the growth medium. Other Esp-deficient strains were shown to produce biofilms, suggesting that Esp is not a critical requirement for biofilm development. Furthermore, his group demonstrated that expression of a secreted metalloprotease, GelE, enhances biofilm formation. Garth Ehrlich presented work on the distributed genome hypothesis. The distributed genome hypothesis states that chronic bacterial pathogens utilize a survival strategy wherein certain contingency genes are distributed among a population and are not found in all members of a species; thus, there exists a supragenome at the population level which is greater than the genome of any one organism. The distribution of contingency genes among a population serves as a supravirulence factor that provides for improved population survival through increased rates of genomic dynamics, which provide for rapid adaptation to environmental conditions through the reassortment of genes. The observation that many bacterial pathogens possess inducible competence and transformation mechanisms, which are activated during times of stress, supports these hypotheses, as do the observations that bacteria in biofilms exchange DNA at much greater rates than do planktonic counterparts.

ORAL BIOFILMS

Perhaps the best-studied model biofilm communities in terms of human health are oral biofilms (57, 83). Hundreds of species have been identified in the oral microbiota (59). Enamel surfaces in the mouth undergo a distinct pattern of colonization or succession. Early colonizers such as *Streptococcus* spp. are followed by secondary colonizers such as *Fusobac-*

terium nucleatum. Specific interspecies interactions drive the colonization process. Species participate in coaggregation and coadhesion events mediated by specific adhesin-receptor interactions. Biofilms are known to contribute to dental caries and gingivitis. There is an appreciation that the composition of the community can dictate disease, as opposed to the presence of a single bacterial species. This has been dubbed "the ecological plaque hypothesis" (70, 71). The oral microbiological community was well represented at this meeting. Annette Moter described her group's efforts to characterize the unculturable organisms associated with periodontal disease (102). They used fluorescent in situ hybridization (FISH) to characterize subgingival biofilms grown in periodontal pockets. The probe EUB 338, specific for the domain *Bacteria*, was used together with a number of species-specific 16S rRNA-directed oligonucleotide probes to identify bacteria. *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, and treponemes of phylogenetic group I were detected with specific probes. This was also the theme of Ann Griffen's presentation. Griffen's group cloned 16S rDNA amplicons from the subgingival pockets of healthy and diseased patients (60): 4,500 clones were identified, and over half of the bacteria detected were uncultivated microbes. Over 75% of the subgingival clones belonged to the phylum *Clostridia*. Of the 19 species statistically shown to be associated with disease in this study, 15 were uncultivated species, while traditionally regarded cultivatable periodontal pathogens such as *P. gingivalis* were detected much less frequently. Her group also reported similar work on dental caries, where again the cultivatable species commonly associated with this disease, including *Streptococcus mutans*, were outnumbered by complex microbial communities, including many uncultivated species.

Coaggregation interactions (cell-cell recognition resulting in clumping between different bacterial species) have been described for the vast majority of oral bacterial isolates. The focus of Rob Palmer's presentation was the evidence that this occurs in vivo. He and his colleagues used immunofluorescence and confocal microscopy to monitor the spatial distribution of possible coaggregation partners on enamel chips retrieved from the human oral cavity. They found that cells reactive with an antibody against RPS (the streptococcal cell surface polysaccharide that is the receptor for protein adhesins on appropriate coaggregation partner cells) and cells reactive with an antibody against a streptococcus that bears appropriate protein adhesins (*Streptococcus gordonii* strain DL1) were closely associated with one another early in biofilm development. They also used an antibody directed against the streptococcus receptor polysaccharide together with an antibody directed against the specific protein adhesin found on *Actinomyces naeslundii* strain T14V to show that streptococcus-actinomyces coaggregation partnerships occur in vivo. Dennis Cvitkovitch presented his work on the role of quorum sensing in biofilm formation in *S. mutans* (17, 66). He initially demonstrated that ComD, a two-component sensor kinase that responds to the competence-stimulating peptide, was defective in biofilm formation. However, a *comC* (the gene encoding the competence-stimulating peptide precursor) mutant had a different phenotype. This prompted an analysis of 13 different two-component systems, identifying 3 that were impaired in biofilm formation and that also responded to the competence-

stimulating factor. Richard Ellen presented his lab's work on the periodontal pathogen *Treponema denticola*. Along with collaborators from Boston and Zurich they found that the outer sheath protease, dentilisin (PrtP), which aids the bacterium's degradation of extracellular matrix, has homologs in several other oral treponemes. The *T. denticola prtP* gene is differentially transcribed in biofilms versus planktonic cells in vitro. The *T. denticola* major outer sheath protein (Msp) dysregulates actin and calcium dynamics in gingival fibroblasts, cells that normally mediate the healing of the extracellular matrix of damaged periodontal tissues.

CORROSION

Biofilms contribute to many industrial processes and problems. Therefore, the ability to control biofilm formation has been an area of intense interest for academic and industrial researchers. Corrosion is one of the hallmark problems caused by biofilms in industry (63). A number of different metabolic groups of bacteria, such as sulfate-reducing bacteria, have been shown to promote corrosion on metal piping. New findings presented at the meeting shed light on how some bacteria cause corrosion and how this process might be controlled. Anne Camper examined multispecies communities and showed that the presence of Fe oxides and humic substances promoted the growth of corrosion-associated biofilm bacteria, while not affecting similar planktonic populations (11, 12). Dianne Newman described how biofilms of dissimilatory iron-reducing bacteria use Fe(III) to respire in the absence of oxygen, which, in some contexts, might protect steel from corrosion (24). She then proposed a model in which redox-active compounds such as phenazines act as extracellular electron shuttles to stimulate Fe(III) reduction under conditions relevant for biofilms (46). Both dissimilatory and nondissimilatory Fe(III)-reducing bacteria can reduce phenazines, and because most phenazines have redox potentials that are lower than those of Fe(III) minerals, this suggests that phenazines may promote microbial mineral reduction in the environment (45). Tom Wood described the use of benign biofilms as a biocontrol strategy to prevent corrosion. Biofilms containing bacteria engineered to produce corrosion inhibitors or antimicrobial compounds active against corrosion-causing bacteria were shown to impede the corrosion process (48, 121). *Bacillus brevis*, secreting gramicidin S, protected pipes from corrosion in the presence of sulfate-reducing bacteria.

MODEL ENVIRONMENTAL BIOFILM SYSTEMS

Another theme of the meeting was the use of model environmental systems to study biofilm communities. One such model system described by Dave Ward was hot spring phototrophic mats (110). These mats represent highly stratified and metabolically integrated communities structured around light and, in certain cases, temperature gradients. These communities are also somewhat stable due to the lack of eukaryotic predation at higher temperatures. In his talk Ward described the distribution and specialization of a key mat community member, the cyanobacteria belonging to the genus *Synechococcus*. He identified several subgroups of *Synechococcus* within these mat communities that have distinct phototrophic

metabolic properties that were indistinguishable from one another at the resolution provided by 16S rRNA-based phylogenetic analysis (29). This finding pointed out one of the limitations of using 16S rRNA as a molecular chronometer and the use of this approach in defining a species. Another interesting result presented by Ward was that although many mat community members showed a high degree of metabolic activity, the growth rates of these organisms were fairly low.

A second model system described at the meeting was wastewater treatment biofilms. These biofilms are extremely important in the wastewater treatment process since they remove many key chemical wastewater contaminants such as nitrogen-containing and phosphorous-containing compounds (77, 108). They also are excellent model systems for examining biogeochemical cycling in microbial communities. Satoshi Okabe presented data showing the distribution of sulfate-reducing bacterial populations in a wastewater biofilm in relation to key redox gradients such as oxygen, nitrate, and sulfate/sulfide, which were spatially resolved by microelectrodes. Using FISH, Okabe's group was able to show that bacteria of the sulfate-reducing genus *Desulfobulbus* were concentrated at the oxic-anoxic interface. Using microautoradiography, he showed that *Desulfobulbus* bacteria were present throughout the biofilm, even in the oxic regions, and were incorporating and oxidizing ^{14}C -labeled propionate (79). Andreas Schramm used a similar multifaceted approach to study the nitrogen cycle in wastewater biofilms. He demonstrated that nitrogen loading levels dictated the types of ammonia-oxidizing and nitrite-oxidizing bacteria that would predominate in sequential batch wastewater biofilm reactors. Both activity and population size increased significantly with higher ammonium concentrations. FISH revealed three distinct ammonia-oxidizing populations, related to the *Nitrosomonas europaea*, *Nitrosomonas oligotropha*, and *Nitrosomonas communis* lineages. This finding suggested that coexistence of these ammonia oxidizers depends upon where they are spatially located in the biofilm in relation to ammonia and oxygen gradients.

ENVIRONMENTAL BIOFILMS AS RESERVOIRS FOR PATHOGENS

There were several laboratories interested in the concept of environmental biofilms harboring pathogenic organisms (30). Timothy Ford suggested that this might constitute a serious problem for immunocompromised individuals using contaminated hospital water supplies. In his presentation, Ford reported that *Mycobacterium avium* complex (MAC) could be isolated from biofilms of hospital hot water systems and these isolates corresponded to the same strains that had infected AIDS patients in the same hospital. He then showed that MAC can be isolated from and thrive within drinking water biofilms and that drinking water is a serious route of exposure to MAC. Along the same lines, a poster presented by G. A. James (G. A. James, R. Hiebert, A. Cunningham, and A. Camper, Biofilms 2003, abstr. 159, 2003) reported that drinking water biofilms exposed to water containing planktonic *S. enterica* serovar Typhimurium, spores of *B. cereus*, and oocysts of *Cryptosporidium parvum* retained these organisms in the biofilm after they were removed from the bulk liquid. Immunoassays detected *S. enterica* serovar Typhimurium 35 days after the biofilms were

exposed to the pathogen, indicating that these organisms persist within the biofilm community.

Genetic exchange between community members in environmental biofilms is another key aspect of environmental biofilms harboring pathogens. The acquisition of new genetic traits may cause a pathogen to become more virulent or more resistant to antimicrobials. Biofilms have been experimentally shown to facilitate conjugal plasmid transfer, and the extracellular matrix of biofilms has been shown to contain nucleic acids as a major constituent. This topic was the subject of a poster presented by Ursula Obst (U. G. Obst, T. Schwartz, and H. Volkmann, Biofilms 2003, abstr. 108, 2003), who reported that a key gene involved in vancomycin resistance, *vanA*, was detected in a drinking water biofilm in the absence of any detectable enterococci, indicating that the biofilm community had the genetic potential to be a source of vancomycin resistance.

Biofilms are also relevant to bioterrorism. Ron Atlas summarized many of the ways indigenous biofilms might be important if biological agents are released into the environment (3, 4). Biofilms could be colonized by pathogens introduced into the environment, potentially complicating detection and decontamination. Another potential issue would be the spread of genes derived from genetically modified pathogens to indigenous species. David White suggested that monitoring drinking water biofilms for toxins and pathogenic microbes would increase detection sensitivity, since biofilms tend to concentrate these agents at their surface (112). He presented data showing that when laboratory biofilms were exposed to pathogens in the bulk liquid, they retained these pathogens and protected them from biocides. Jon Calomiris (Biofilms 2003, abstr. 170, 2003) developed a model laboratory system to grow municipal water-fed biofilms on copper pipes. Copper tubing in the presence and absence of biofilms was subjected to a pulse of *Bacillus anthracis* (ATCC 4229) spores. The number of spores attached to the biofilm was an order of magnitude higher than the number attached to the naked copper surface, suggesting that biofilms may concentrate spores. These studies strongly indicate that an examination of native biofilms would be key to understanding the fate of pathogens released into the environment.

EMERGING TECHNOLOGIES FOR STUDYING HETEROGENOUS COMMUNITIES

One of the distinct benefits of attending this meeting was to see how scientists representing different disciplines try to study biofilm communities. What are commonly used and effective tools for studying biofilms in one field may be conspicuously absent in another. In the context of wastewater biofilms, Michael Wagner demonstrated how environmental microbiologists determine what organisms are present, where they are localized in relation to key chemical gradients, and which metabolic activities they are participating in. In an impressive display of technology, Wagner's group analyzed wastewater biofilms and determined the (i) spatial resolution of individual bacterial species and phylogenetic groups (using FISH), (ii) profiles of key chemical gradients (using microsensors), and (iii) types of metabolic activity in the community (using microautoradiography-stable isotope probing) (Fig. 3) (1, 18, 36, 64). Clinical researchers are just now starting to regularly employ

FIG. 3. Combining FISH with microautoradiography. Confocal laser scanning microscopic images of artificial mixtures of *E. coli* and *Herpetosiphon aurantiacus* incubated with [3 H]glucose and analyzed by a combination of microautoradiography and FISH by using Cy3-labeled probe GAM42a (red) and Cy5-labeled probe EUB338 (colored green by image analysis). (Top left) Microautoradiographic image of *E. coli* and *H. aurantiacus* after 3 h of incubation with [3 H]glucose. (Bottom left) Whole-cell hybridization of the microscopic field in the top left panel. *E. coli* cells appear yellow because of the overlapping labels. (Top right) Microautoradiographic image of *E. coli* and *H. aurantiacus* after 3 h (*E. coli*) and 24 h (*H. aurantiacus*) of incubation with [3 H]glucose. (Bottom right) Whole-cell hybridization of the microscopic field in the top right panel. (Reprinted with permission from *Applied and Environmental Microbiology* [64]).

these tools to study medical biofilms and will probably gain new insight into biofilm infections as a result. Thomas Neu presented his group's work using fluorescently labeled carbohydrate-specific lectins to analyze EPS distribution in complex environmental biofilms (7, 75). In conjunction with confocal microscopy, Neu used the full range of commercially available lectins to evaluate the presence of biofilm-specific glycoconjugates as well as the volume of biofilm cellular and polymeric constituents. He showed that changing the carbon source fed to these biofilms impacted EPS glycoconjugate composition.

The P pilus is an important adhesin of uropathogenic *E. coli*. Jana Jass described the use of optical tweezers and atomic force microscopy to measure the adhesive properties of the P pilus of *E. coli*. She reported strong interactive forces (~ 150 pN) between recombinant *E. coli* heterologously expressing the P pilus and polystyrene beads. These forces were absent when the beads were incubated with P pilus-negative *E. coli*. Interestingly, in the presence of specific P-pilus receptors, the interactive forces were an order of magnitude weaker. Her work also indicated that the P pilus exhibits a great deal of flexibility.

Another challenge in studying biofilm communities is the inability to assess gradients of gene or protein expression in a heterogenous system. Almost every study to date that has conducted a DNA microarray or proteomic analysis of a biofilm

population had to take an average expression profile for the entire population. Marvin Whiteley described applying a variation of the recombination in vivo expression technology-based approach to *P. aeruginosa* PA14 in order to assess expression profiles (90). This system is based upon creating genomic libraries in which the DNA fragments are fused at random upstream of a promoterless gene encoding the TnpR recombinase. By using *sacB* selection, if TnpR is expressed *P. aeruginosa* will not grow on sucrose. This approach can be used to identify promoters expressed in a biofilm. By varying the translational efficiency of *tnpR* by modifying the Shine-Dalgarno sequences, promoters with a range of strengths can be identified. Although this analysis is just beginning, Whiteley's approach highlights a new genetic tool that should eventually allow dissection of the heterogeneity present in biofilms.

BIOFILM FORMATION AS DEVELOPMENT—FITTING A SQUARE PEG INTO A ROUND HOLE?

Significant discussion centered on whether biofilm formation is a form of differentiation and a form of "true" development. The strict definition of a differentiation process would be a state where other environmental inputs are ignored in order to proceed through a programmed series of structural and/or behavioral changes. The concept of vectoral checkpoints, through which the organism cannot reverse its course of differentiation, is a central concept of development. This paradigm is based on metazoan developmental pathways that are largely controlled through temporal and structural checkpoints (e.g., *Hox* genes of *Drosophila*) (13). There are clearly examples of prokaryotic differentiation, including endospore development in *Bacillus* and *Clostridium* (84, 96), fruiting body and spore formation in myxobacteria, and stalk-to-swarm transition in *Caulobacter* (51, 82, 84). These examples satisfy most if not all the criteria used to define classical developmental processes.

The general features and three-dimensional complexity achieved in some biofilms has led to the idea that biofilm formation is a developmental process, with true differentiation of at least some of the cells within a biofilm into a new, nonplanktonic state. Given the definition of development and differentiation, this would require that cells within the biofilm enter into a rigid path towards the sessile, biofilm-specific state, including the successful transit of checkpoints along the way (see reference 10 for such a definition). The differentiation of these cells should at some point be independent of additional environmental inputs. While there are certainly some examples of such fixed, metazoan-type development in prokaryotes, biofilm formation in most systems does not appear to meet all of the criteria described above. In most cases, the process is still strongly influenced by prevailing environmental conditions (flow properties, nutrient conditions, etc.) and is reversible at many steps along the way. Individual cells can dissociate from the biofilm apparently at any time. Likewise, biofilms are readily colonized by planktonic cells virtually at any point. Given such observations, it seems that the consideration of microbial biofilms in terms of metazoan development may miss the target. Defining experimental approaches and evaluative criteria based on metazoan-type development may ignore or miss some of the important features and emer-

gent properties of biofilm communities. Furthermore, restricting our studies and conceptual framework in terms of development is probably selling biofilms short.

If we accept that most biofilm formation is not true development, does this mean that it is simply single-cell physiology of multicellular proportions? Undoubtedly, the answer to this question is no. There are clearly emergent properties of biofilms that distinguish them from single cells. Antimicrobial resistance, diffusion-gradient-dependent growth patterns, distributive behaviors such as quorum sensing, and microscale phenotypic radiation are properties of biofilms that are not or cannot be reproduced by single cells. Biofilms are clearly more than the sum of their parts. Biofilm formation also exhibits directionality, if not absolute checkpoints. Processes that exhibit hysteresis, where there is a greater barrier to moving in reverse than moving forward, clearly occur within biofilms. Once a structurally complex biofilm begins to form, the likelihood of all the cells of that biofilm reverting to a planktonic state is clearly less likely than continued accumulation of the biofilm. Although the process is reversible, there is directional momentum. Therefore, while true checkpoints may not exist or at least be rare in microbial biofilm development, directionality in biofilm formation seems a certainty. The inherent flexibility in biofilm formation is in fact one of the key attributes of these multicellular structures and distinguishes them from the lock-step developmental pathways of metazoan organisms. It seems that we are in a situation where most biofilm formation is more than simply adaptive physiology as defined for single cells but not quite a true developmental process. Complexity is introduced by the structure, chemical and physical heterogeneity, and differential genetic potential of the biofilm constituents. In some cases, distributive behaviors, such as quorum sensing, may further solidify the behavior of the biofilm as a single unit. If we hope to understand biofilms to the point where we can manipulate their formation, final architecture, and eventual dissolution, we must recognize and appreciate their unique position across the spectrum of cellular behaviors.

Much progress has been made in this area since the last biofilm meeting, changing the general disposition of the field. There was a sense at the 2000 biofilm meeting in Big Sky, Mont., that biofilm development was a fixed program that could ultimately be explained and controlled through genetic analyses. The paradigm for biofilm development at the time was based mainly upon the study of *P. aeruginosa*. In this developmental scheme single cells attached to a surface, multiplied, moved together by twitching motility to form small aggregates or microcolonies, and ultimately grew to form mature biofilms characterized by mushrooms of bacteria encased in EPS. Each of these steps was thought to require the indispensable contribution of different gene-encoded functions. The literature at the time reinforced this notion, and eventually the point was reached that if you weren't growing mushrooms, there was something terribly wrong (Fig. 4). Much research was conducted and reported in the format of "factor X is required for biofilm development." The truth is that although there are mutants that are severely impaired in forming biofilms, no one factor has yet been identified that is absolutely required. Findings presented at the Victoria meeting indicate there is a growing appreciation for the multifactorial nature of biofilm formation.

FIG. 4. "The perfect mushroom" Micrograph represents a three-dimensional image of a *P. aeruginosa* PAO1 biofilm grown in a flow cell reactor. The biofilm field has propagated the perception that all species march through a program of gene expression to form mature biofilms with this characteristic structure. Much data presented at the meeting contradicts this: biofilms come in all shapes and sizes, and their architecture is as much a consequence of the environment as it is of gene expression. Cells are expressing GFP. Magnification, $\times 630$. Image courtesy of Ehud Banin, University of Iowa.

CONCLUSION

The field of biofilm research has progressed tremendously since the previous Biofilms conference in 2000. One of the major themes to emerge from this conference was that of diversity. The increased diversity of different labs performing sophisticated studies on biofilms and the range of new approaches in use to examine microbial biofilms are particularly striking. Also striking is the diversity of microorganisms that are being studied in the context of biofilms. The range of microbial assemblages that are considered to be biofilms has undergone diversification since the previous meeting, now embracing structures such as pods and host-associated aggregates in addition to those structures previously accepted as representing biofilms. Findings presented in Victoria additionally reveal the diverse mechanisms by which microbes can associate with interfaces and with each other, under a range of conditions. One of the major reasons that biofilm research has progressed so rapidly in recent years is the advent of more sophisticated and more standardized physiological, microscopic, genetic, and molecular approaches for their study. This is a process that needs to continue in order for the field to maintain its considerable momentum.

Among all of this diversity however, the Biofilms 2003 conference made it abundantly clear that the field of biofilm research still requires of a certain level of unification. The Biofilms 2003 conference highlighted the things we do not know as much as those that we do. The field of biofilm research is still in its early stages. Many of the complex processes that dictate biofilm formation and stability are just now being identified, and far from completely understanding the process, we are to some extent still gathering the pieces of the puzzle before beginning to assemble them into a coherent whole. The com-

mon threads and mechanisms shared among many if not all biofilms are just now coming to light, through much of the work presented at this conference. However, a great deal of research remains to be done in order for the biofilm puzzle, with all of its myriad essential pieces, to come together. Along the way, the findings generated from this field of investigation will continue to provide practical benefits and conceptual insights into many aspects of biology, biotechnology, and medicine. We look forward to the continued emergence of this unique and inherently multidisciplinary branch of microbiology over the coming years and expect the next Biofilms conference to foster the same excitement and high-level scientific discourse as its predecessors.

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