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The biofilm community resurfaces: new findings and post-pandemic progress

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ABSTRACT The ninth American Society for Microbiology Conference on Biofilms was convened in-person on 13–17 November 2022 in Charlotte, NC. As the first of these conferences since prior to the start of the COVID-19 pandemic, the energy among the participants of the conference was clear, and the meeting was a tremendous success. The mixture of >330 oral and poster presentations resoundingly embodied the vitality of biofilm research across a wide range of topics and multiple scientific disciplines. Special activities, including a pre-conference symposium for early career researchers, further enhanced the attendee experience. As a general theme, the conference was deliberately structured to provide high levels of participation and engagement among early career scientists.

KEYWORDS biofilms, conference review

The American Society for Microbiology (ASM) convened its ninth Conference on Biofilms in Charlotte, NC in November 2022 (11/13–17). This was the second focused ASM conference since the COVID-19 pandemic began and was postponed from 2021 in response to rising SARS-CoV2 infections in that year. The 2022 conference was, in general, a tremendous success, with a large number of biofilm researchers in attendance and a palpable spirit of enthusiasm to share their findings and obtain feedback from the community. This review first describes the conference format and special features followed by summaries of the oral presentations.

CONFERENCE STRUCTURE AND TOPICS COVERED

Overall, the ninth ASM Conference on Biofilms was attended by over 400 participants, with 55 full oral presentations (22 invited and 33 selected from submitted abstracts), plus 12 short lightning talks, in 11 thematic sessions. These sessions were (i) natural biofilms, consortia, and simulations; (ii) initiating contact: signals and mechanisms promoting attachment; (iii) biofilm maturation pathways, consequences, and control; (iv) synthesis, assembly, and function of extracellular biofilm determinants; (v) metabolism, physiology, and structure of biofilms; (vi) susceptibility and tolerance: antimicrobials and biofilms; (vii) social and asocial interactions in biofilms, (viii) signals and mechanisms promoting biofilm disassembly and dispersal; (ix) host-associated biofilms, (x) control, prevention, and elimination of biofilms, and (xi) innovative approaches and new technologies in biofilms research. Outstanding keynote talks from Jean-Marc Ghigo (Institut Pasteur, Paris, France) and Robin Patel (Mayo Clinic, Rochester, MN, USA) were bookends to the conference. There were 336 posters presented in four separate sessions, representing an enormous breadth of exciting biofilm research. Conference chair Karen Visick and co-chair Clay Fuqua worked together with an outstanding organizing committee composed of biofilm researchers (Table 1). The 2022 conference offered several new features

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TABLE 1 Conference organizing committee

Committee member	Institution
Karen Visick, Chair	Loyola University Chicago, Maywood, IL, USA
Clay Fuqua, Co-chair	Indiana University, Bloomington, IN, USA
Ehud (Udi) Banin	Bar-Ilan University, Ramat Gan, Israel
Alain Filloux	Nanyang Technological University, Singapore
Maria Hadjifrangiskou	Vanderbilt University, Nashville, TN, USA
Susanne Häußler	Helmholtz Centre for Infection Research, Braunschweig, Germany
Birthe Veno Kjellerup	University of Maryland, College Park, MD, USA
Dean Rowe-Magnus	Indiana University, Bloomington, IN, USA
Kendra Rumbaugh	Texas Tech University Health Sciences Center, Lubbock, TX, USA
Mark Schembri	University of Queensland, Queensland, Australia
Paul Stoodley	Ohio State University, Columbus, OH, USA

BOX 1. LIGHTNING TALKS

One of the new features incorporated into the ninth Conference on Biofilms was the addition of Lightning talks, three-minute oral presentations from early career scientists. A set of three lightning talks were presented each day, permitting another 12 participants—and their lab groups—to be highlighted to conference attendees over the 4-day meeting. Given that the biofilm field continues to expand, this feature provided an opportunity for a wider range of community members to be recognized and spotlighted and a more diverse array of topics to be included. Lightning talk presenters gave an overview of their work and invited the audience to learn more by visiting their posters later in the day. In some ways, the Lightning presenters had the best of both worlds: an opportunity to address the conference audience as a whole, garnering their interest, and the ability to engage one-on-one during the poster session.

relative to previous conferences, including the new Lightning Talk sessions (Box 1). A highly successful Early Career Symposium (ECS) was held on the afternoon of Sunday, 13 November, organized by an Early Career Committee chaired by Jennifer Greenwich and Derek Fleming and composed of graduate students and postdoctoral scientists (Table 2). It featured two guest keynote talks and nine oral presentations from speakers selected from the conference abstracts (see Early Career Symposium Box 2). The ECS committee also organized multiple other career and networking activities during the full conference. The conference also offered a highly subscribed Biofilm Imaging Workshop on the afternoon of Tuesday, 15 November (Coordinators: Paul Stoodley, Ohio State University, Columbus, OH, USA; Erin Gloag, Virginia Tech., Blacksburg, VA, USA; Hannah Jeckel and Knut Drescher, Biozentrum—University of Basel, Switzerland), providing hands-on guidance on COMSTAT and Biofilm Q packages for image analysis and processing. The conference also featured a Diversity, Equity, and Inclusion event on the evening of Wednesday, 16 November, in which the documentary film "Picture a Scientist" was screened to stimulate an open dialogue on equity and diversity in biofilm research and microbiology in general (Discussion Leaders: Maria Hadjifrangiskou, Vanderbilt University, Nashville, TN, USA; Dean Rowe Magnus, Indiana University, Bloomington, IN, USA; Julia van Kessel, Indiana University, Bloomington, IN, USA). Several social events provided excellent opportunities for networking and collaborative interactions. Overall, these new features were considered by the conferees to be highly positive and to add to the conference.

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TABLE 2 Early career committee

Committee member	Institution	Career stage
Jennifer Greenwich, Co-chair	Indiana University , Bloomington, IN, USA	Postdoctoral scientist
Derek Fleming, Co-chair	Mayo Clinic, Rochester, MN, USA	Postdoctoral scientist
Sheyda Azimi	Georgia Institute of Technology, Atlanta, GA, USA	Postdoctoral scientist
Courtney Dial	Loyola University Chicago, Maywood, IL, USA	Graduate student
Shahrzad Saffari Ghandehari	University of Maryland, College Park, MD, USA	Graduate student
Fabrice Jean-Pierre	Dartmouth College, Hanover, NH, USA	Postdoctoral scientist
Benjamin Wucher	Dartmouth College, Hanover, NH, USA	Graduate student

BOX 2. EARLY CAREER SYMPOSIUM

The inaugural Mark E. Shirtliff Biofilms ECS was held prior to the start of the main ASM Conference on Biofilms. This symposium benefitted from generous sponsorship from the Mark Shirtliff Biofilm Foundation, as well as other sources. The ECS brought together close to 90 graduate students and postdoctoral researchers for an afternoon of oral presentations and networking opportunities. In addition to selected abstract presentations from graduate students and postdoctoral researchers, two keynote talks were presented by Fitnat Yildiz (University of California, Santa Cruz, CA, USA) and Sophie Darch (University of South Florida, Tampa, FL, USA). Both speakers highlighted their career trajectories and research projects, describing how they established their own laboratories and navigated their way to their current positions.

Abstract speakers presented their findings on a variety of topics ranging from fundamental aspects of cell biology to the immune response to infection. Two speakers focused on the oral microbiome and periodontal disease: Zhi Ren (laboratory of Hyun (Michel) Koo, University of Pennsylvania, Philadelphia, PA, USA) spoke on interkingdom biofilms formed by *Candida albicans* and *Streptococcus mutans* in human saliva, which are highly correlated with extensive tooth decay in children, and Justien Ghesquière (laboratory of Kristel Bernaert, KU Leuven, Leuven, Belgium) reported on spatiotemporal changes to multispecies biofilms in response to prebiotic treatments. Three of the talks featured *Pseudomonas aeruginosa*: Courtney Kleeschulte (laboratory of Matthew Parsek, University of Washington, Seattle, WA, USA) discussed a new model for studying biofilm dispersal by stopping media flow in the flow biofilm model; Nasibeh Arabameri (laboratory of Boo Shan Tseng, University of Nevada, Las Vegas, NV, USA) described her efforts to study biofilm heterogeneity by using single-cell RNA sequencing to develop transcriptomic profiles of individual cells; and Nicole Fazio (laboratory of Courtney Reichhardt, Washington University, St. Louis, MO, USA) presented her investigations of the interaction between the adhesin protein CdrA and the Psl polysaccharide in *P. aeruginosa* biofilms.

The remaining four abstract speakers covered a diverse range of topics. Amelia Staats (laboratory of Paul Stoodley, Ohio State University, Columbus, OH, USA) discussed the immune response to prosthetic joint infections and how aggregates of *Staphylococcus aureus* induce IL-8 expression in synovial fluid. Aggregation of the marine bacterium *Alteromonas* in response to dipepetides was the focus of Jacob Robertson (laboratory of Terence Hwa, University of California, San Diego, CA, USA). James Winans (laboratory of Carey Nadell, Dartmouth College, Hanover, NH, USA) described the protective effect of biofilm aggregates against phage exposure for *Escherichia coli* and *Vibrio cholerae*. Ian Reynolds (laboratory of Clay Fuqua, Indiana University, Bloomington, IN, USA) detailed his findings on the plant pathogen *Agrobacterium tumefaciens* and the interplay between lipopolysaccharides, flagellar motility, and polar surface attachment. Zhi Ren and Ian Reynolds were chosen

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by the Early Career Committee for best presentation awards, which also enabled them to present their work at the full ASM Conference on Biofilms. Overall, the ECS conference was considered a great success by the attendees and clearly helped to impart palpable energy and focus on training and development for the whole conference.

The study of biofilms has generated a revolution in the way that we view microbial life. Biofilms are the culmination and complex manifestation of microbial processes that have been well studied over the past century, but these multicellular assemblies force us to consider microbial activity in a more realistic context, in many cases resulting in unexpected, emergent properties. In a practical sense, biofilms have a tremendous economic impact, in multiple sectors of human activity including health, agriculture, and industry (1). From the basic science perspective, biofilms offer a wealth of new discoveries, expanding the breadth and reach of microbiology. The ninth ASM Conference on Biofilms covered a wide range of biofilm-related research, from the fundamental processes that underlie their formation and dissolution, to their impact on the environment and on human health, to the development of new approaches to mollify or harness their activities (Fig. 1). This review attempts to capture the tremendous energy and enthusiasm the conference generated and describes some of the new and exciting science that was presented.

KEYNOTE ADDRESS: JEAN-MARC GHIGO

The opening keynote speaker for the ninth ASM Conference on Biofilms was Jean-Marc Ghigo from the Institute Pasteur (Paris, France) who gave an intriguing presentation suggestively entitled "*Biofilm* $^{Al}chem_{y}{}^{i}st_{e}ry$." Among the many scientific contributions across a wide range of biofilm research from Ghigo and co-workers, this presentation highlighted several noteworthy findings that have changed, or are changing the way we view biofilms. An important goal was to address the question of what properties are truly biofilm specific. One of the emergent attributes of biofilms that greatly impacts society and medicine is their increased tolerance toward antimicrobial treatment. In efforts to define biofilm-specific processes in *Escherichia coli,* the Ghigo group discovered that the lipid A component of lipopolysaccharide is highly palmitoylated in biofilms (2). LPS modification does not affect biofilm formation *per se* but rather enhances the tolerance toward certain antibiotics and can diminish the inflammatory response in mammalian hosts. Conditions within biofilms lead to increased osmotic stress, activating the RcsB system (independent of its well-characterized role as a phosphorelay), which in turn increases expression of *pagP*, encoding an enzyme that palmitoylates the lipid A moiety (3). Continuing to explore which processes are characteristic of biofilms, the Ghigo lab developed a clever cultivation scheme in which volatile compounds released from *E. coli* biofilms cultivated in micro-fermentation vessels were trapped by a resin, and fractions were then analyzed by gas chromatography-mass spectrometry. A strong signal from the compound 1-propanol was identified and revealed activation of an amino acid fermentation pathway, induced by anaerobic conditions that develop within biofilms (4). The 1-propanol is generated from the fermentation of the amino acid threonine, a process activated under low oxygen, enabling oxidation of reduced NADH pools. This pathway had not been identified prior to this work in *E. coli,* and, thus, this biofilm-triggered metabolic pathway revealed a new process, occurring in conditions created by the biofilm environment, that imparts a strong fitness benefit to biofilm cells (4). Ghigo and colleagues, among others, have in fact characterized several bacterial volatile compounds (BVCs), such as ammonia, trimethylamine, and hydrogen cyanide, which can allow the producer cells to promote long range interactions with other bacteria, plants, and animals (5). As an intriguing illustration of the potential for these air-borne BVC pathways, Ghigo described findings in which mice could respond to olfactory signals

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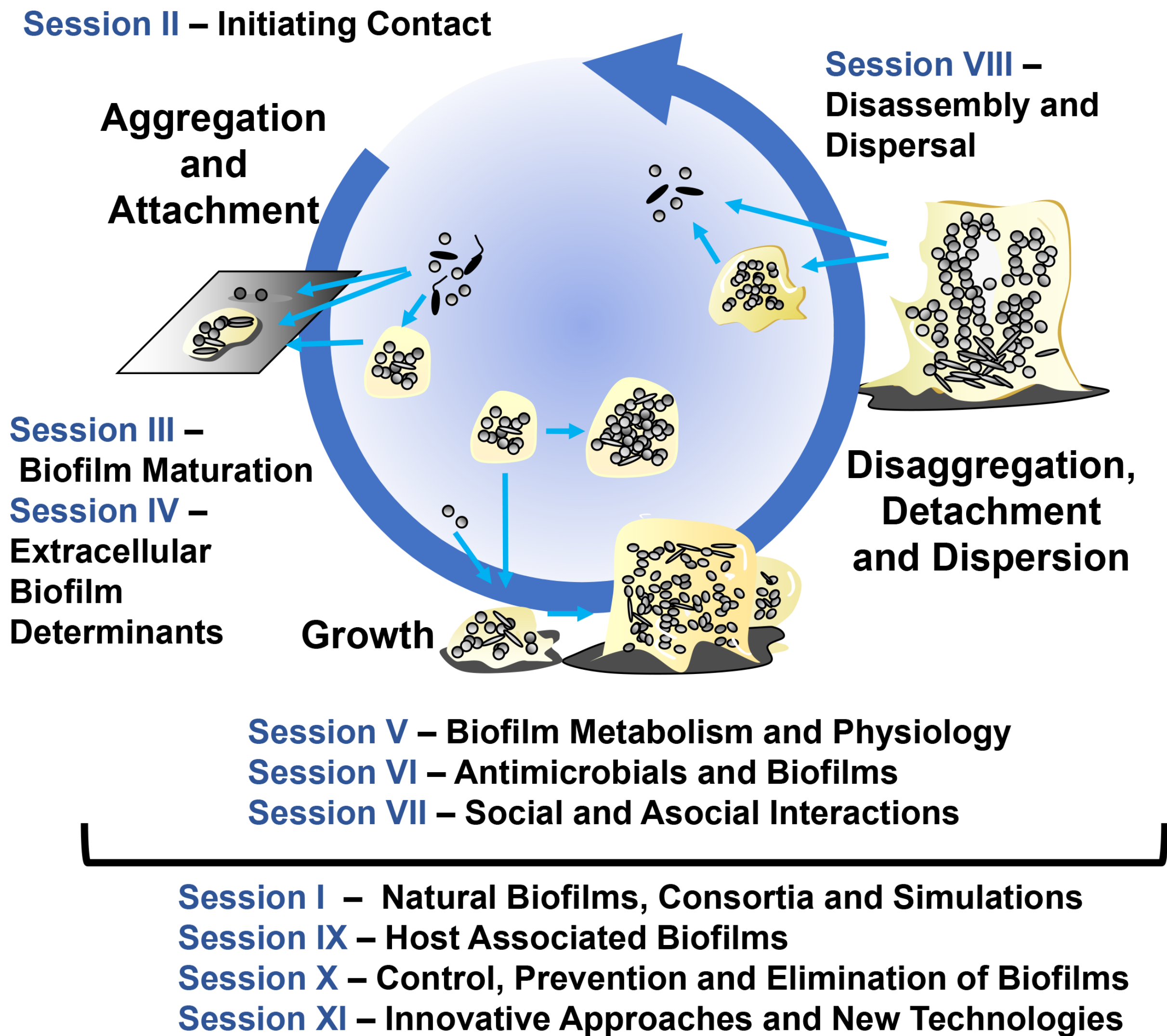

FIG 1 Model of the biofilm life cycle, with multiple microbial species indicated by different cell shapes. Conference sessions specifically relevant to one portion of the life cycle are arrayed in the appropriate position, whereas other sessions that applied to biofilms overall are indicated below the horizontal brackets.

from *Pseudomonas aeruginosa* biofilms, suggesting that bacterial biofilms may alter animal behavior through BVCs.

The importance of oxygen limitation and anaerobiosis in many biofilm-specific processes has been established for multiple bacterial taxa. To explore a fully anaerobic system, the Ghigo lab has investigated *Bacteroides thetaiotaomicron*, an obligate anaerobe that is quite abundant in the mammalian enteric system, as a model for biofilm formation (6). Although the most widely studied laboratory strain of *B. thetaiotaomicron* is a poor biofilm formed *in vitro*, mutations that lead to biofilm formation have revealed a role for a Type V secretion system and capsular polysaccharide in surface adhesion (7, 8). A major signal for biofilm formation in multiple isolates of *B. thetaiotaomicron*, including the lab strain, is the presence of bile, which induces expression of multiple genes including a DNA nuclease gene (*bipD*), mutations of which result in decreased biofilm formation (9). These and related studies are beginning to reveal those functions that are unique to biofilm formation in obligate anaerobes and to what extent they overlap with those from biofilms formed under aerobic conditions.

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NATURAL BIOFILMS, CONSORTIA, AND SIMULATIONS

Invited speaker Raymond Hozalski (University of Minnesota, St. Paul, MN, USA) started the session with a talk that covered "real-world" biofilms in water distribution pipelines, where biofilms comprise >95% of the total number of bacteria and even higher levels in drinking water main pipes. The two main topics discussed were: (i) presence of opportunistic pathogens and (ii) nitrification and chloramine decay for disinfection processes. The samples used in this study were very challenging to collect since they were obtained from large water storage towers/tanks as well as underground water main pipes during repairs. Chloramine is used as disinfectant in the United States to maintain the water quality. During the fall season, the chloramine broke down, decreasing its concentration and releasing ammonia, which caused ammonia oxidizing bacteria (AOB) to flourish and produce nitrite. As the nitrite can react with chloramine to form nitrate and release more ammonia, this results in an acceleration in chloramine decay. *Mycobacterium* spp. were identified in the systems, which, fortunately, were not the species involved in infectious diseases, and *Legionella* spp. were only observed in drinking water systems without residual disinfectant concentration (10, 11). Dr. Hozalski concluded that drinking-water biofilms are complex communities that, among other microorganisms, can harbor the nuisance AOB, and at lower levels *Legionella* and *Mycobacterium*.

The focus then shifted from drinking water systems to anadromous and freshwater fish, in a presentation from Kelly Crandall (laboratory of Kat Milligan-McClellan, University of Connecticut, Storrs, CT, USA). Her work was predicated on the increasing levels of plastic in water worldwide, which provide a large surface area where biofilms can form and potentially degrade the plastics. In Crandall's study, 350 bacterial isolates from fish were evaluated and several produced enzymes that may be useful for the biodegradation of different types of plastics. Using the three-spined stickleback as a model system, the biodegradation of several plastic types by bacterial isolates from these fish was evaluated. These isolates showed preference for specific types of plastic, potentially reflecting different adhesive mechanisms responsible for biofilm formation.

Invited speaker Alan Decho (University of South Carolina, Columbia, SC, USA) discussed how biofilms can form in environments with transient water presence, such as in soil crusts and deserts, rather than the saturated environments in the previous presentations. Bacteria can survive desiccation due to extracellular EPS components in the biofilm. Rehydrated cells can resynthesize macromolecules and strengthen the EPS matrix structure. The hypersaline microbial mat systems in the Bahamas are an example. The mats experience alternating dry and wet seasons, are photosynthetic, have a high microbial diversity (including archaea), and have very abundant EPS in their surface layers. As the EPS dries, it condenses and a hard "plastic"-like film forms, where cells can be observed inside together with vesicles and cell capsules. This "organic glass" preserves the extracellular biomolecules and can prevent denaturation. Dr. Decho suggested that these structures were not only relevant for natural life on Earth but maybe also on non-Earth planets such as Mars, where salt brines have been identified.

Courtney Dial (laboratory of Karen Visick, Loyola University Medical Center, Maywood, IL, USA) presented her studies of *Vibrio fischeri*, the specific symbiont of the light organ of the Hawaiian bobtail squid *Euprymna scolopes* (12). In this symbiosis, transient biofilms form on the surface of the light organ that promote subsequent colonization. For wild-type *V. fischeri*, biofilm formation under standard laboratory conditions is weak, although specific regulatory mutants form robust biofilms through the production of the Syp polysaccharide. Exploration of media conditions revealed that $CaCl_2$ and the vitamin para-aminobenzoic acid (PABA) could coordinately stimulate biofilm formation by the wild-type strain through increased cyclic diguanylate monophosphate (c-di-GMP) levels and Syp production (13). Subsequently, the sensor kinase RscS was identified as a key regulator responsible for promoting biofilms in response to the PABA/$CaCl_2$ conditions (14). The identification of these key signals is a major step forward in understanding the complex control over biofilm formation during symbiotic colonization of the light organ.

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Zhi Ren (laboratory of Hyun "Michel" Koo, University of Pennsylvania, Philadelphia, PA, USA) was one of the two awarded ECS speakers who was selected to present his work to the larger conference audience (Table 3). Dr. Ren showed visually stunning microscopy of an interkingdom assemblage in saliva of toddlers with tooth decay, consisting of the bacterium *Streptococcus mutans* and the pathogenic yeast *Candida albicans*. These microorganisms establish structured assemblages when they are present in human saliva, which can colonize dental surfaces (15). The fungal clusters carried the associated *S. mutans* and displayed a leaping-type motion while continuously growing (Fig. 2). The assemblages were "lifted" from the surface and expanded laterally but still retained contact at some positions, resulting in a rudimentary but dramatic form of surface locomotion to quickly spread on the tooth surface. This complex microbial growth pattern may exacerbate tooth decay due to increased acid production at the biofilm-dental interface.

INITIATING CONTACT: SIGNALS AND MECHANISMS PROMOTING ATTACHMENT

The shift from a free-living existence to a surface-attached or aggregated conformation is a major transition for microorganisms and typically is a necessary first step of biofilm

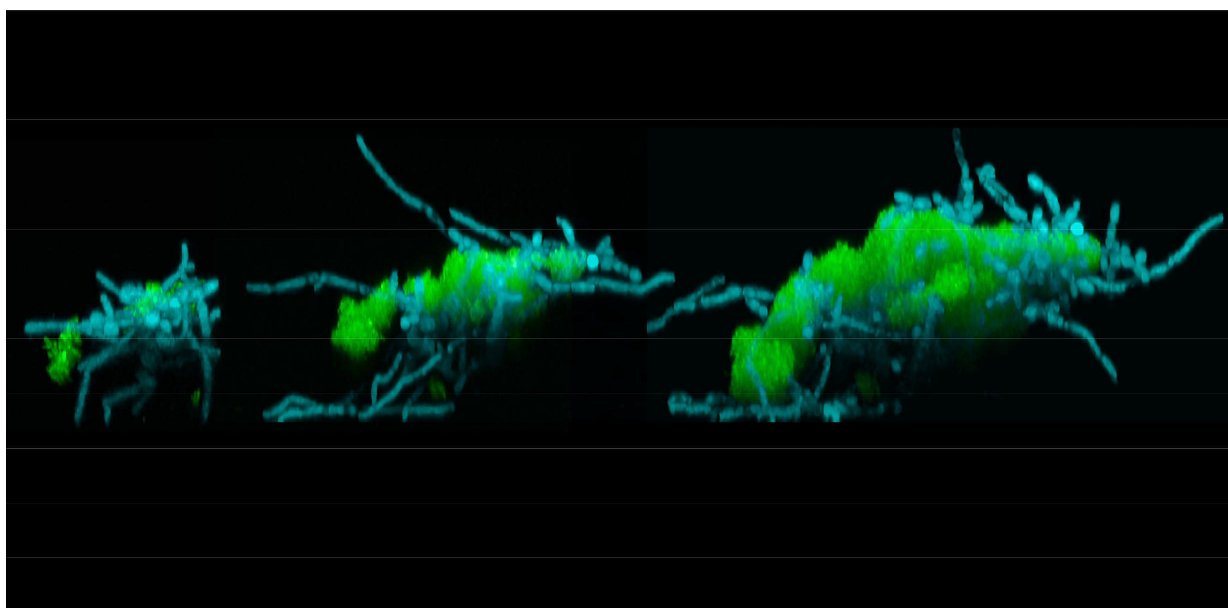

FIG 2 *Candida-Streptococcus mutans* co-aggregate. A "leaping-like" motion as fungi (in blue) propel bacteria (in green) forward along the tooth surface. The image was created by stitching together selected frames from a confocal time series to better illustrate different stages of locomotion. [Image courtesy of Zhi Ren and Hyun (Michel) Koo, reproduced with permission.]

TABLE 3 Conference awards

Awardee	Career stage	Institution	Sponsor	Award
Amber Hendricks	Postdoctoral scientist	Nationwide Children's Hospital, Columbus, OH, USA	NPJ Biofilms and Microbiome Award	Oral presentation
Hamilton Green	Graduate student	Vanderbilt University, Nashville, TN, USA	FEMS Microbiology Reviews	Poster and lightning
Chita Ravi	Graduate student	University of Queensland, Queensland, Australia	Biofilms Elsevier	Oral presentation
Kailyn Jessel	Graduate student	University of Michigan, Ann Arbor, MI, USA	Biofilms Elsevier	Oral presentation
Zhi Ren	Postdoctoral scientist	University of Pennsylvania, Philadelphia, PA, USA	ECS Symposium	Oral presentation
Ian Reynolds	Graduate student	Indiana University, Bloomington, IN, USA	ECS Symposium	Oral presentation

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formation. Multiple structures on the cell surface can promote these interactions, and this session focused on these structures and how they are regulated. Invited speaker Courtney Reichhardt (Washington University, St. Louis, MO, USA) started off the session with a description of recent work from her lab on the remarkable fibrillar adhesin protein CdrA of *Pseudomonas aeruginosa*. This large protein is secreted to the cell surface where it can interact with other CdrA molecules as well as specifically binding the polysaccharides Psl and Pel and impacting *P. aeruginosa* biofilm structure (16, 17). CdrA has a modular structure, and the Reichhardt lab is using a variety of approaches to study its conformation. Their findings suggest that CdrA structure is quite different from that predicted through homology modeling, with an elongated, rigid structure comparable to a matchstick with a globular domain on the end of the filament extended well away from the cell surface. The mechanism by which CdrA associates with the different polysaccharides and potentially other extracellular components remains under investigation. Xuhui Zheng (laboratory of Matthew Parsek, University of Washington, Seattle, WA, USA) also focused on *P. aeruginosa*, specifically differential responses at surfaces. She compared fully submerged biofilms to those at air-surface interfaces using fluorescent reporters for both cyclic-AMP (cAMP) and c-di-GMP second messengers. Production of cAMP induced by the Pil-Chp system promotes twitching motility, whereas c-di-GMP drives sessile biofilm formation (18). Submerged biofilms in flow cells caused increases in c-di-GMP, whereas cAMP levels were not affected, in contrast to biofilms on agarose pads exposed to air where a steady increase in cAMP was observed. This work reveals how *P. aeruginosa* can respond in different ways to these two environments.

Two talks in this session examined surface colonization in the *Alphaproteobacteria* group, the prosthecate aquatic microbe *Caulobacter crescentus* and the model plant pathogen *A. tumefaciens*. David Hershey (University of Wisconsin, Madison, WI, USA) has been examining the role of *C. crescentus* flagellar genes and their role in surface sensing. *C. crescentus* has a biphasic life cycle, and newly released motile daughter cells (often called swarmer cells) carry a single polar flagellum produced at the old pole (19). During surface colonization, the flagellum is lost, and the adhesive holdfast is extruded at the end of a specialized polar extension of the cell wall called the stalk. The holdfast can adhere cells to surfaces, and surface interactions can accelerate holdfast production (20). Rotation of the flagellum plays a role in activating holdfast biogenesis (21). Hershey and colleagues found that mutating genes for flagellar assembly also caused acceleration of holdfast production, connecting flagellar status with holdfast production. A suppressor screen for mutations that reverse the increased holdfast production of specific flagellar mutants identified a number of flagellar signaling suppressor (*fss*) mutants. The *fss* mutations map to genes required for other flagellar biosynthesis genes, stalk production, and cell-cycle control. The work reported is beginning to reveal the regulatory architecture that leads from the flagellum to the holdfast biosynthetic machinery in *C. crescentus*. Jennifer Greenwich (laboratory of Clay Fuqua, Indiana University, Bloomington, IN, USA) described her studies on control of the adhesive polar UPP polysaccharide by a regulatory mechanism that requires metabolites known as pterins (22, 23). The pterin response is mediated through a c-di-GMP signal transduction pathway and the DcpA dual function diguanylate cyclase-phosphodiesterase. DcpA activity is regulated by pterins released to the periplasmic space and bound by the PruR pterin binding protein, which forms a complex with the DcpA periplasmic domain. Greenwich et al. have interrogated this pathway in *A. tumefaciens*, including structural characterization of the PruR-pterin interaction. The PruR-DcpA regulatory circuit is encoded in a single operon, and this module is conserved with multiple pathogens in the *Proteobacteria*, suggesting a common role for pterin-responsive regulation.

Invited speaker Diana Morales (Weill Cornell Medical College, New York, NY, USA) presented findings from her lab on the Gram-positive pathogen *Enterococcus faecalis*. Following colonization of surfaces in the gastrointestinal tract, *E. faecalis* can translocate through the host epithelial layer and spread to other systems. Translocation requires production of a *N*-acetyl glucosamine (GlcNAc)-containing polysaccharide that drives

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aggregation of *E. faecalis* (24). Using agar penetration as a model for tissue colonization, Morales and co-workers have performed elegant microscopic examination of these surface-penetrating cell populations, revealing changes in the thickness and lipid composition of the envelope including increased amounts of glycolipid, most markedly diglucosyl-diacylglycerol (DGDAG) (Fig. 3) (25). Expression profiling of these populations suggests that they activate a distinct transcriptional program that includes restructuring of the envelope. These translocating populations also exhibit increased tolerance toward multiple antibiotics, perhaps contributing to the difficulty in treating infections that break through the gastrointestinal tract.

BIOFILM MATURATION: PATHWAYS, CONSEQUENCES, AND CONTROL

Although surface attachment is a requisite first step to biofilm formation, many of the emergent properties of biofilms arise from subsequent maturation of the biofilm after the initial stages. This session explored the processes and properties that lead to later stage biofilms, subsequent to attachment. Invited speaker Kai Thormann (Justus-Liebig University, Giessen, Germany) presented novel work on the regulation of the *Shewanella putrefaciens* surface adherence. PdeB is a multidomain hybrid protein that degrades the intracellular messenger c-di-GMP and is localized to the flagellated cell pole via direct interaction between its inactive diguanylate cyclase domain and the FimV domain of the polar landmark protein HubP (26). The restriction of the phosphodiesterase activity of PdeB to the flagellated cell pole results in c-di-GMP concentration heterogeneity, which orchestrates the population-level behavior with respect to cell-surface interaction and environmental spreading.

DNA is a well-established component of the biofilm matrix in certain bacterial species. Amber Hendricks (laboratory of Steven Goodman, Nationwide Children's Hospital, Columbus, OH, USA) reported on the recent discovery, obtained using several different bacterial pathogens, that the conformation of biofilm extracellular DNA (eDNA) shifts from the canonical B-form to the novel Z-form as the biofilm matures (27). Z-form DNA

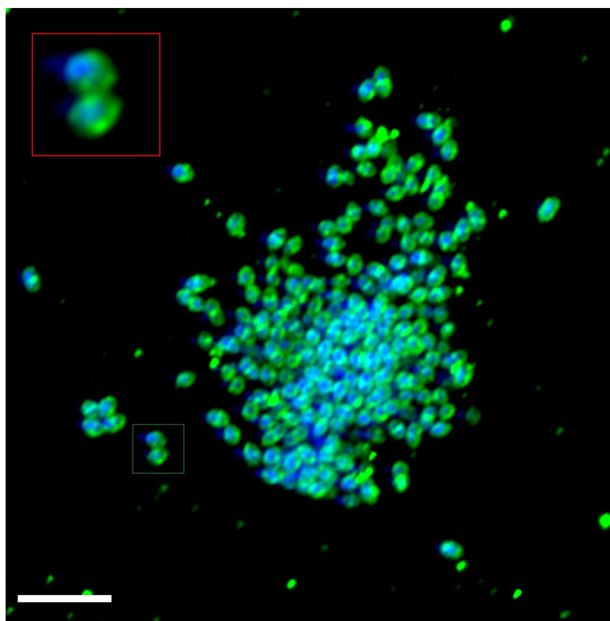

FIG 3 Population of *Enterococcus faecalis* penetrating a surface. Fluorescence microscopy using BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) staining (green) revealed areas of neutral lipid accumulation at the cell envelope (inset) of *Enterococcus faecalis* surface-penetrating aggregates. This was supported by mass spectrometry analysis, which showed increased levels of total DGDAG in this population (25). The bacterial DNA was visualized by DAPI staining (blue). Scale bar: 4 µm. (Image courtesy of Diana Morales and Yusibeska Ramos, reproduced with permission.)

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is recalcitrant to association with B-DNA binding proteins, including H-NS, and decreases its interaction with biofilm eDNA. Interestingly, released exogenous H-NS was demonstrated to prevent the formation of neutrophil extracellular traps (NETs) when induced, such as with phorbol myristate acetate. Thus, while H-NS does not have a structural role in mature biofilms, it appears to have a role in defending biofilm-grown bacteria from host-derived NETs due to DNA condensation of NET-deployed DNA. Dr. Hendricks's excellent presentation was recognized with an award from NPJ Biofilms and Microbiomes (Table 3).

Exopolysaccharides and protein fibrils can also play critical roles in biofilm maturation. Invited speaker Lynette Cegelski (Stanford University, Palo Alto, CA, USA) described her group's work with solid-state NMR to investigate how the biological functions of cell walls and biofilms depend on their chemical composition and architecture. She reported the genetic and molecular basis for the installation of a newly discovered chemically modified cellulose (phosphoethanolamine cellulose) produced by *E. coli* that, together with curli amyloid fibers, confers remarkable community architectures (28, 29). Ongoing chemical and genetic approaches promise to identify potentially novel bacterial polysaccharides as well as novel inhibitors of these bacterial biofilm matrix components.

Biofilm structure can also have practical consequences in an applied context. The bacterium *Geobacter sulfurreducens* is a major platform for bioenergy production. Dr. Katy Juarez (Biotechnology Institute, National Autonomous University Mexico, UNAM, Cuernavaca, Mexico) elucidated the connection between biofilm formation and a key transcriptional regulator encoded by the *gsu1771* gene (30). *G. sulfurreducens* can acquire energy by coupling oxidation of organic compounds with extracellular electron transfer to different insoluble electron acceptors through *c*-type cytochromes and conductive pili. Electroconductivity was found to be increased 100-fold in a *gsu1771* null mutant. RNA-seq experiments from glass-grown biofilm and mobility shift assays confirmed the role for GSU1771 in the regulation of genes involved in exopolysaccharide production and signal transduction, as well as genes involved in energy metabolism and electron transfer.

The final architecture of biofilms is determined by a complex combination of environmental conditions and microbial cell biology. Hannah Jeckel (laboratory of Knut Drescher, Biozentrum, University of Basel, Switzerland) applied a computational framework to quantify and compare the biofilm architecture of different bacterial species in both experimental and mechanistic mathematical modeling approaches (31). The two control parameters of cell-aspect ratio and cell density were found to have a major impact on biofilm architecture for multiple bacterial species. Systematic variations of these parameters in *Vibrio cholerae* mutants confirmed a causal relationship and led *V. cholerae* to form biofilm architectures that are characteristic of biofilms of other species. Furthermore, the parameter variations using mutants also enabled successful prediction of biofilm architectures in mechanistic simulations, thus demonstrating that mechanical cell-cell interactions dominate early biofilm development.

SYNTHESIS, ASSEMBLY, AND FUNCTION OF EXTRACELLULAR BIOFILM DETERMINANTS

Building on the themes from the prior session, speakers in this session focused on the variety of extracellular components that can comprise the biofilm matrix and how the cells synthesize and construct the matrix. The most common components in the matrix include a variety of polysaccharides as well as proteins and nucleic acids. The speakers in this session discussed the biosynthesis of these components individually and how they interact together to form the biofilm matrix.

The interplay and regulation between large surface proteins can have a major impact on the formation of the matrix and biofilms. Invited speaker Alex Horswill (University of Colorado School of Medicine, Aurora, CO, USA) began the session with a discussion of *Staphylococcus aureus* biofilms. Previous work has shown that the two-component

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system ArlRS regulates clumping and that mutants are unable to aggregate due to failure to interact with fibrinogen (32). ArlRS indirectly inhibits elaboration of multiple large surface proteins including Ebh [extracellular matrix (ECM)-binding protein homologue] and SasG (*S. aureus* surface protein G) through activation of the inhibitor MgrA. Mutants in *arlRS* and *mgrA* are deficient for immune evasion functions and elevated for aggregation, and Δ*mgrA* null mutants clump in human saliva due to elevated levels of SasG (33, 34).

Released nucleic acid, most notably eDNA, can also contribute to biofilm matrix formation and structural integrity. Sudarsan Mugunthan (Nanyang Technical University, Singapore) described an examination of how nucleic acids function within the extracellular matrix as biopolymers. Noncanonical base pairing and G-quadruplex eDNA structures form within the matrix of *P. aeruginosa* biofilms, providing mechanical stability and imparting viscoelastic properties (35). RNase treatment with DNAse pre-treatment disrupted biofilms, RNA was detected directly within the biofilm matrix, and RNA transesterification in response to elevated pH led to loss of matrix integrity, suggesting roles for released RNA along with DNA in this context.

It is well established that polysaccharides also play a central role in the biofilm matrix. Bacteria will often modify polysaccharides prior to export and incorporation into the matrix. For example, poly-*N*-acetylglucosamine (PNAG), a common component in the biofilm matrix for multiple microorganisms, must first be partially deacetylated before export in Gram-negative bacteria. Adithya Subramanian (laboratory of Lynne Howell, The Hospital for Sick Children and University of Toronto, Toronto, Canada) presented findings on the mechanisms of PNAG synthesis in *E. coli*, specifically the role for the tetratricopeptide repeat domain in the PgaA outer membrane porin that is responsible for export of PNAG. PgaA interacts with the PgaB enzyme, functioning as a scaffold and modulating its activity. PgaB is both a deacetylase and a glycoside hydrolase, with the deacetylase activity increasing upon interaction with PgaA, which in turn binds PNAG for export (36).

Cellulose is another common polysaccharide component of the biofilm extracellular matrix of bacteria. Chitra Ravi (laboratory of Mark Schembri, University of Queensland, Brisbane, Australia) described findings on the biosynthesis of cellulose in Uropathogenic *Escherichia coli* (UPEC) and the comprehensive identification of genes involved in the regulatory pathway through a transposon mutant screen. Of the 96 independent genes identified, many of them are involved in cellulose biosynthesis and export. Additionally, disruption of the lipid A core component of LPS impaired cellulose production, as did mutations of genes that control purine and pyrimidine biosynthesis genes, potentially through disrupting biosynthetic precursor pools. Both a diguanylate cyclase and a phosphodiesterase were identified, consistent with a role for c-di-GMP in this process. This excellent talk and comprehensive genetic analysis were awarded one of two early career awards from the Elsevier *Biofilms* journal (Table 3). The genetic detail provided in this talk was further extended in the second invited talk by Petya V. Krasteva (University of Bordeaux, Pessac, France). Bacterial cellulose (BC) is simultaneously synthesized and secreted into the periplasm, where it can be modified prior to export. Among different bacteria, there are several variations on the common machinery composed of the c-di-GMP-sensing cellulose synthase BcsA and its partner protein BcsB, with different accessory proteins, some requisite for cellulose production (37). Krasteva described remarkable structural insights that detail how different types of BC are secreted via the Bcs system, using *E. coli* and the cellulose overproducer *Gluconacetobacter hansenii* as models. It was thought that BcsA and BcsB interact strictly in a 1:1 heterodimeric complex. Cryoelectron microscopy has, however, revealed that in *E. coli* BcsB multimerizes with as many as six subunits as a “crown” on a single BcsA protein within the context of the larger biosynthetic macrocomplex. Accessory proteins contribute in different ways. As an example, the ATP binding and hydrolysis activity of BcsQ, stabilized by the small protein BcsR, are required for secretion, whereas together with BcsE the three contribute for cyclase-proximal c-di-GMP enrichment (38, 39). The crystalline character

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of the *G. hansenii* cellulose, on the other hand, appears to be imparted through the linear alignment of multiple Bcs subcomplexes and a cytosolic BcsH-BcsD scaffold (40). A remarkable level of detail is emerging on how different cellulose machineries function among bacteria and to what extent these are similar to cellulose biosynthetic mechanisms in plant systems.

METABOLISM, PHYSIOLOGY, AND STRUCTURE OF BIOFILMS

It is clear that biofilms dramatically change the cellular activity of their constituents, impacting the emergent properties of these populations. Speakers in this session focused on ways to stimulate the metabolism of biofilm cells to reduce antimicrobial tolerance, the physiology of subpopulations, the localization and aggregation of dual species biofilms, and models recreating infection-relevant biofilms *in vitro*.

Bacteria living in a biofilm are generally characterized by reduced metabolic rates, with metabolic adaptations involving decreased tricarboxylic acid cycle activity, decreased proton-motive force, and decreased uptake, but increased activity of enzymes linked to the glyoxylate shunt (41). However, active bacterial metabolism is a prerequisite for optimal activity of many classes of antibiotics. Leading off the session, invited speaker Tom Coenye (Ghent University, Ghent, Belgium) explored the link between metabolic adaptations in biofilms and reduced antibiotic susceptibility by determining whether stimulating microbial metabolism in biofilm cells diminishes antimicrobial tolerance. His group made use of *P. aeruginosa* aggregates, formed in an artificial cystic fibrosis (CF) sputum medium, to measure the potentiating effects of carbon sources on the efficacy of ciprofloxacin (CIP) and ceftazidime, using microcalorimetry to measure metabolic rate. Several carbon sources primarily linked to the tricarboxylic acid cycle, including citric acid, malic acid, and sodium acetate, enhanced antibiotic efficacy in killing *P. aeruginosa* aggregates while also increasing ROS (reactive oxygen species) production (42). Specific carbon sources also increased killing of *P. aeruginosa* while decreasing tissue cell death for biofilms grown in a 3D lung cell culture model, as measured by decreased lactate dehydrogenase (LDH). The results suggest that stimulating metabolism can potentiate the efficacy of antibiotics under biofilm growth conditions.

Nutritional effects such as carbon and nitrogen abundance also influence aggregate formation and the metabolic adaptation of the aggregated cells. Using the nontuberculous mycobacterial species *Mycobacterium abscessus* as a model and the microbial identification passive clarity technique-hybridization chain reaction (MiPACT-HCR) technique that preserves the three-dimensional structure of aggregates within tissue (43), invited speaker Will DePas (University of Pittsburgh, Pittsburgh, PA, USA) demonstrated that aggregate formation was dependent on carbon source type and availability, similar to *Mycobacterium smegmatis* (44). In particular, abundance of glucose enhanced *M. abscessus* aggregation, while nitrogen abundance (through glutamine and ammonium) decreased aggregation by favoring dispersal and growth as planktonic cells. Moreover, oxygen availability was required for both aggregation and dispersal. Inhibiting protein synthesis with chloramphenicol suppressed the transition between aggregates and planktonic cells in a manner similar to exposure to an anaerobic environment.

Oxygen availability was also a major theme for Kailyn Jessel (laboratory of Matthew Chapman, University of Michigan, Ann Arbor, MI, USA). She described how *E. coli* that normally forms rugose colonies bifurcates into two distinct subpopulations within biofilms, one type that forms rugose colonies, producing an ECM composed of curli fibers and cellulose polymers, and a second type lacking an ECM. The two subpopulations differed in the expression of genes involved in motility, curli production, environmental sensing, anaerobic respiration, and stress responses. Moreover, biofilms grown under anaerobic conditions had little to no ECM production compared to biofilms grown under aerobic conditions, as determined by lack of Congo red binding, lack of curli-producing cells, and decreased production of CsgA and CsgB curli proteins. Addition of nitrate partly restored rugose biofilm morphology to cells that were grown

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anaerobically. Jessel's excellent presentation was recognized with an award from Elsevier *Biofilms* journal (Table 3).

Dual species biofilms were featured in two presentations in this session. Pathogens *Stenotrophomonas maltophilia* and *P. aeruginosa* are often co-isolated from the lungs of CF patients. Stefan Katharios-Lanwermeyer (laboratory of Anupama Khare, National Institutes of Health, Bethesda, MD, USA), reported on the mechanism by which *P. aeruginosa* induces aggregation in *S. maltophilia*. Aggregate formation by *S. maltophilia* was found to be an active process and induced by *P. aeruginosa* cell-free supernatant. The *P. aeruginosa*-secreted factor was shown to be indirectly controlled by rhamnolipids with work ongoing to elucidate its specific nature. Aggregation by *S. maltophilia* required the presence of a fimbrial adhesin encoded by the *smf*-1 gene.

Dual species biofilms between bacteria and yeast were described by Dr. Katherine Baxter (laboratories of Gail McConnell and Paul Hoskisson, University of Strathclyde, Glasgow, United Kingdom), exploring the large-scale interactions of *Staphylococcus aureus* and *Candida albicans*. Using the Mesolens 3D imaging system that allows subcellular resolution for a large area of biofilm under low magnification, Baxter was able to visualize overall biofilm architecture (45). When inoculated onto agar, mixed cultures of *S. aureus* and *C. albicans* form a central core of *C. albicans* interspersed with punctate *S. aureus* microcolonies. Later stage biofilms develop a corona of *S. aureus* at the colony periphery apparently corralling the *C. albicans/S. aureus* central core.

SUSCEPTIBILITY AND TOLERANCE: ANTIMICROBIALS AND BIOFILMS

As highlighted so well in the keynote presentation by Jean-Marc Ghigo, increased tolerance toward antimicrobial therapies is one of the most impactful emergent properties of biofilms. Invited speaker Oana Ciofu (University of Copenhagen, Copenhagen, Denmark) provided a comprehensive overview of tolerance and for development of resistance in biofilms (46). She demonstrated how the response of biofilms depends on the antibiotic mode of action, the sub-population being considered, and the biofilm age. The dynamics that foster emergence of resistance differ between biofilms and dispersed planktonic bacteria. Recent work suggests that the resistance to antibiotics develops faster in biofilms than in planktonic cultures, enabling the emergence of a greater diversity of new variants adapted to the biofilm mode of growth, with different fitness requirements compared to planktonic cultures. Thus, biofilms represent a potential reservoir for the development of mutational resistance. Using ciprofloxacin (a DNA gyrase inhibitor) and *P. aeruginosa*, it was elegantly revealed that the antibiotic induces ROS production, which increased mutation rates, creating greater likelihood for efflux pump activation. After long exposure to ciprofloxacin, resistance in biofilms emerged more rapidly than that observed in planktonic cultures, although planktonic bacteria were resistant to much higher concentrations. Interestingly, combining the antibiotic treatment with an antioxidant slowed the occurrence of mutation, suggesting the use of this approach as an anti-evolutionary strategy, which may be effective for treating biofilms.

Vaughn Cooper (University of Pittsburgh, Pittsburgh, PA, USA) continued the discussion of antibiotic resistance evolution within biofilms (47). Using *Acinetobacter baumannii* as a model, Francine Arroyo and Cooper combined antibiotic treatment with CIP and the beta-lactam ceftazidime (CEF), two antibiotics for which sensitivity is reciprocally collateral, with CIP resistance often resulting in greater sensitivity to CEF. The effects on therapy with the individual drugs were compared to combined therapy for 15-day-old biofilms vs planktonic cultures in a bead-based biofilm cultivation format, and deep sequencing was used to identify the underlying mutational spectrum. Consistent with the observations cited by Oanu Ciofu (described above), the mutational diversity was higher in biofilms, but the overall MIC was considerably lower than that emerged in planktonic cultures. A Pareto optimality front describes a condition in which a resulting increase in resistance comes at the cost of increased sensitivity to the other drug. The team applied a Pareto-type model to summarize the collateral effects of

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both mono- and combination therapies. Trade-offs were apparent with exposure to the individual antibiotics, whereas the combination therapy was less subject to these trade-offs. Interestingly, mutation order was also important, and high-level resistance required multiple mutations. Under all conditions, antibiotic treatment selected for mutations in the *adeIJK*-encoded efflux pump, although initial driver mutations in efflux pump regulators and phospholipid modification systems differed between biofilm and planktonic populations. The results highlight the use of Pareto models to describe emergence of multi-drug resistance.

The complex selective pressures imposed during clinical infections can differ significantly from these laboratory models. As an example, Amanda Morris (Hospital for Sick Children, Toronto, Canada) described the correlation of aggregation and Psl exopolysaccharide production in clinical sputum samples with eradication failure of *P. aeruginosa* in children with CF (48). Using the MiPACT approach on sputum samples and fluorescent *in situ* hybridization targeting *P. aeruginosa* in parallel with anti-Psl antibody fluorescent labeling, elevated Psl labeling was observed in persistent infection samples and in isolates tested *in vitro*. Although it was an understandably small sample size, the general trend was that the samples from CF children that failed tobramycin therapy had more *P. aeruginosa* aggregates with a greater biovolume and higher Psl production (normalized for *P. aeruginosa* biovolume) compared to the children who responded to the antibiotic therapy. These results highlight utilization of direct visualization for infecting bacteria in clinical samples to predict the outcome of antibiotic therapy.

A very different perspective on antibiotic tolerance was presented by Maria Van Rossem (University of Southampton, Southampton, UK), who focused on the development of a model to characterize antibiotic tolerance in liquid-crystalline biofilms (49). Liquid crystals (LC) are formed in *P. aeruginosa* biofilms by filamentous bacteriophage Pf4 virions in combination with extracellular matrix components. Van Rossem et al. developed an *in vitro* model of the Pf4 virion in which phages co-align to form rugby ball-shaped LC droplets termed tactoids. The tactoids engulfed the bacteria and were shown to act as a shield protecting these cells against antibiotics. The neutralizing activity was observed for cationic antibiotics and was attributed to the physical barrier provided by the LCs and the anionic nature of the phages, which absorb the antibiotic and reduce its diffusion. The result corroborates a previous work that showed that Pf4 virions increase tolerance to cationic antibiotics (50).

Another surprising mechanism contributing to the antibiotic tolerance of biofilms was described by invited speaker David Andes (University of Wisconsin, Madison, USA), who focused on the role of biofilm matrix components of the pathogenic yeast *Candida*. *Candida* is a common cause of medical device-associated infections, and matrix components have been shown to sequester antifungal agents (51). Apparently, three major polysaccharides act synergistically to establish the *Candida* biofilm matrix, and strains that have decreased matrix or lack one of the polysaccharides, also have lower antimicrobial tolerance. The matrix is thought to be assembled after all the components are secreted from the cell, often within extracellular vesicles (EVs) (52). Comparing EVs from planktonic and biofilm cultures, they observed that biofilm cells secreted much smaller EVs that contained matrix components. Mutants in vesicle formation created a deformed matrix and were more sensitive to antimicrobials, but these properties could be rescued by the addition of exogenous wild-type vesicles. Finally, treatment with turbinimicin (which inhibits vesicle formation) combined with other antifungal agents showed a synergistic effect that is related to the damage of the extracellular matrix. Taken together, the results suggest that inhibition of EV formation may be an attractive antibiofilm drug target.

SOCIAL AND ASOCIAL INTERACTIONS IN BIOFILMS

Many biofilms contain multiple species of bacteria, with complex associations that create heterogeneity across biofilms (53). This session was led off with invited speaker Mette Burmølle (University of Copenhagen, Copenhagen, Denmark), who described that

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the emergent properties of a biofilm community composed of four co-isolated soil strains, *Stenotrophomonas rhizophila*, *Xanthomonas retroflexus*, *Microbacterium oxydans*, and *Paenibacillus amylolyticus*. Past work had revealed that these strains produced more biofilm together than as individuals, exhibiting cooperative interactions that depend on the presence of all four organisms (54, 55). Recent studies indicate that these strains produce specific patterns of spatial organization as well as synergies in several behaviors such as swarming motility. Excitingly, this group of organisms, but not individual taxa alone, appeared to protect plants against the effect of drought conditions.

Further delving into the theme of bacterial interactions, Carey Nadell (Dartmouth College, Hanover, NH, USA) posed the questions of how cell behavior in biofilms affects group architecture and how the resulting structure correspondingly affects the ecology of the system (56). Using the predatory microbe *Bdellovibrio bacteriovorus* and *V. cholerae* as prey, the Nadell group has determined that biofilms formed by *V. cholerae* protect against *Bdellovibrio* attack, which is dependent on the structure of the biofilm and the matrix protein RbmA. When biofilms comprising mixtures of *V. cholerae* and *E. coli* were exposed to *B. bacteriovorus*, *V. cholerae* protected *E. coli*, but in doing so became somewhat more sensitive to predation. Protection depended on the initial distance between the two organisms, with a closer starting distance resulting in a well-mixed biofilm and a disrupted structure that was poorly protective, whereas a greater starting cell-cell distance permitted *V. cholerae* to grow into protective structures that envelop small groups of *E. coli*, which are then protected as well.

Biofilms form using secretions of polysaccharides and other molecules that are deemed "public goods" as they can be shared among participating organisms. However, their production is tightly controlled, often by quorum-sensing mechanisms, because these secretions are costly to produce, and non-producers or "cheaters" can take advantage of the resources without contributing. Kathleen O'Conner (laboratory of Steve Diggle, Georgia Institute of Technology, Atlanta, GA, USA) addressed the problem of how bacteria shape their world by probing the interactions of *P. aeruginosa* strains with different cell surfaces (having altered hydrophobicity dependent on whether they contain or lack their surface O-antigen) under conditions that mimic those found in CF lungs (57). By modifying growth conditions to alter the reliance of bacteria on quorum-sensing-controlled public goods, this group found that similar cells (with the same surface hydrophobicity) would group together as asocial entities unless forced, by the need to use public goods, to overcome negative surface interactions, thus driving production of well-mixed social structures.

P. aeruginosa evolves rapidly in the CF lung, resulting in an array of variants, including those with increased mucoidy due to overproduction of the polysaccharide alginate. Erin Gloag (Virginia Tech, Blacksburg, VA, USA) explored the potential benefit(s) to *P. aeruginosa* of co-existing with other strains with altered mucoidy in a mixed biofilm. Confocal microscopy and creep-recovery analyses indicated that the resulting mixed biofilm comprised non-mucoid microcolonies that are formed on top of the mucoid cells adhered to the substratum, and exhibited increased biomass and increased viscoelasticity, phenotypes that may represent an advantage to *P. aeruginosa* in the context of the CF lung.

Interactions with macrobiota can also strongly influence the interaction of biofilm constituents, and conversely these interactions can impact activity on the host. Invited speaker Danielle Garsin (University of Texas, Houston, TX, USA) closed out the session by describing tripartite interactions between the nematode *Caenorhabditis elegans*, the bacterial pathogen *Enterococcus faecalis*, and the pathogenic yeast *C. albicans*. While both bacterial and fungal pathogens rapidly kill *C. elegans*, a mixture of the two resulted in slower killing, similar to exposure to a non-pathogen. Further investigation revealed that *E. faecalis* secretes a small peptide, EntV, that inhibits hyphal development and biofilm formation by *C. albicans in vitro* and reduces fungal tongue infection in an oropharyngeal candidiasis mouse model (58). Structural analysis of EntV revealed that it has a clasping palm structure that protected a 16-amino acid central helix that

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comprised the active component (59). Work is underway to develop a minimally sized derivative as an antifungal therapeutic.

SIGNALS AND MECHANISMS PROMOTING BIOFILM DISASSEMBLY AND DISPERSAL

A great deal of research focuses on preventing biofilm formation. However, it is not always possible to prevent this process, and, therefore, there is a high level of interest in triggering biofilm dispersal and other ways to promote biofilm disassembly. Invited speaker Karin Sauer (Binghamton University, Binghamton, NY, USA) kicked off the session by describing her elegant work on the regulation of biofilm dispersion for *P. aeruginosa*. She made the analogy that the biofilm matrix forms a protective bunker, and dispersal mechanisms must deconstruct this bunker. The biofilm can rapidly disassemble, and this is at least partially due to environmental cues that activate functions, which effectively degrade the matrix, such as polysaccharases and nucleases. These biofilm disassembly functions are highly regulated through a complex c-di-GMP network responsive to environmental cues such as nitric oxide and involving a chemotaxis-type transducer protein BdlA and the AmrZ regulator (60). Interestingly, as *P. aeruginosa* cells are dispersed, they enter into a specialized mode of growth that Sauer and colleagues consider a disseminative state, distinct from planktonic and biofilm cells. This work nicely reinforces the concept that dispersal is a programmed phase of the biofilm life cycle (Fig. 1).

Similar to the environmental control of dispersal in *P. aeruginosa*, Francisco Albicoro (laboratory of Çagla Tükel, Temple University, Philadelphia, PA, USA) described how the host metabolites lactate and nitrate, produced during intestinal infections with *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), also act to trigger dispersal of *S*. Typhimurium biofilms (61). Inflammation is caused in response to these bacteria, as well as to curli β-amyloid fibers produced by *S*. Typhimurium biofilms. Albicoro and colleagues found that nitrate and lactate reduce curli and cellulose production in a dose-dependent manner, with lactate having the most pronounced effect. This dispersal activity was traced to decreased expression of *csgD*, encoding a biofilm master regulator, and in turn the *csgBAC* curli operon. Accordingly, the reduced expression of these genes was found to be tied to low intracellular cyclic-di-GMP levels in response to the presence of these signals, overall leading to dispersal of *S*. Typhimurium biofilms.

The interactions of many bacterial pathogens with host tissue surfaces can be impacted by chemical modification of adhesive polysaccharides. Multiple strains of *Staphylococcus epidermidis* utilize the poly *N*-acetyl glucosamine polysaccharide known as PIA (polysaccharide intercellular adhesin) to adhere to surfaces, including to fibrin deposited onto the surfaces of indwelling medical devices. J. Scott VanEpps (University of Michigan Medical School, Ann Arbor MI, USA) presented the studies of Shannon VanAken, a research specialist in his lab, on PIA-mediated interactions with fibrin using an *in vitro* fibrin-coated model surface. The IcaB protein acts to deacetylate PIA, modifying its adhesiveness and interaction with fibrin and with other *S. epidermidis* cells. Null mutation of *icaB* leads to less aggregation of *S. epidermidis* and more interaction with the fibrin. The greater fibrin association results in more rapid degradation of the fibrin network via proteolysis, which can change the clotting properties of the fibrin. Introduction of a protease inhibitor can slow fibrin degradation. In sum, this study demonstrates how multiple interactions and dispersal mechanisms can impact the host interactions of *S. epidermidis* and by extension other staphylococci with host surfaces.

Two of the talks in this session focused on multispecies interactions and their impact on the resulting biofilms, including their tendency to disperse. The lungs of CF patients often foster polymicrobial infections, and examples of bacteria that co-exist in this environment are *P. aeruginosa*, *S. aureus*, and nontuberculous mycobacterial pathogens such as *Mycobacterium abscessus*. In the presentation from Ayantu Idosa (laboratories of Luanne Hall-Stoodley and Daniel Wozniak, Ohio State University College of Medicine, Columbus, OH, USA), she described how in dual-species colony biofilm cultivation, *M.*

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absceccus is severely antagonized by *P. aeruginosa*, even though this is not observed in planktonic culture, thus suggesting a surface-dependent antagonism (62). *P. aeruginosa* mutants in known surface-contact-dependent and diffusible inhibitor pathways retained this antagonistic activity, so this appears to be a new inhibitory mechanism. Multiple strains of *P. aeruginosa* manifest this activity, and other mycobacteria, such as *Mycobacterium smegmatis* are also inhibited.

The interactions between *P. aeruginosa* and *S. aureus* have been well studied, given their established frequent co-infections in CF patients (63). Invited speaker Dominique Limoli (University of Iowa, Iowa City, IA, USA) presented new findings on the response of *P. aeruginosa* to phenol-soluble modulins (PSMs), peptide cytotoxins produced by *S. aureus*. *P. aeruginosa* is activated for twitching motility over solid surfaces by the PSMs, effectively enveloping the non-motile adherent *S. aureus* aggregates (64). This motile response involves the Pil-Chp chemosensory-pilus biogenesis apparatus, which drives Type IV pilus-mediated chemotaxis toward the source of the PSMs. Limoli shared striking microscopy that reveals these dynamic interactions during biofilm formation (Fig. 4). Additionally, proteomic analysis suggests that the PSMs activate the Type VI secretion system of *P. aeruginosa* and pyoverdine production. Described as "interspecies weaponry," Limoli hypothesized that the response to PSMs represents a form of "competition sensing," with *P. aeruginosa* apparently adopting the strategy that the best defense is a strong offense to dominate in these competitions. On surfaces, *P. aeruginosa* generates a hostile environment against many competing bacteria, through direct attack as well as more clandestine forms of competition such as iron sequestration to disperse or inactivate the biofilms of its competitors.

HOST-ASSOCIATED BIOFILMS

It is abundantly clear that biofilms in the context of a living host organism manifest properties that can differ dramatically from biofilms on abiotic surfaces (65). Furthermore, many bacterial populations have a significant fraction of single cells as well as multicellular aggregates and biofilms. This session focused specifically on these host-associated bacterial assemblies. Invited speaker Thomas Bjarnsholt (Costerton

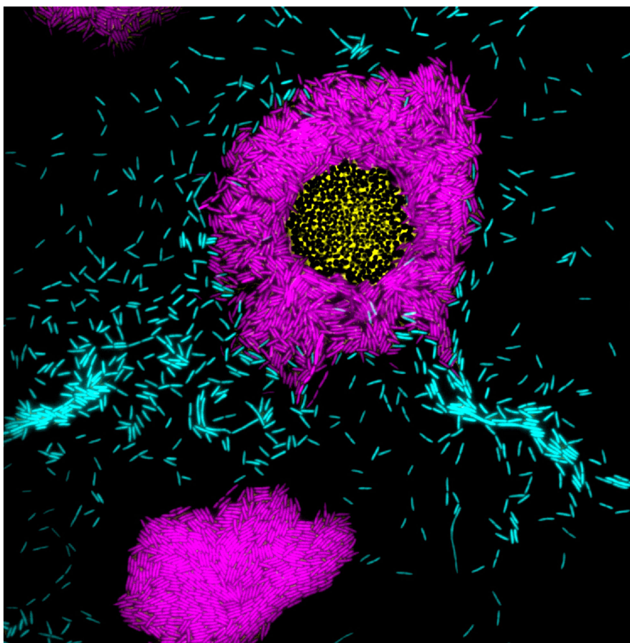

FIG 4 *Pseudomonas aeruginosa* cells labeled magenta are capable of sensing and attacking *Staphylococcus aureus,* labeled yellow, while *P. aeruginosa* cells in cyan have been genetically modified to be blind to interspecies signals. (Image courtesy of Dominique Limoli, reproduced with permission.)

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Biofilm Centre, University of Copenhagen, Copenhagen, Denmark) described the potential for chronic infection as a combination of pathogen inoculum density, a set of risk factors for each host, and the infectious micro-environment (IME). He provided evidence that both biofilm-associated cells and single cells can be found at chronically infected human sites, as well as during acute infections (66). The role of these infection site-associated single cells in the progression of chronic infection and resistance to therapy, their differences from single planktonic cells, and the impact of the IME remain to be determined. The micro-environment of chronically infected sites is shaped by various physicochemical factors as well as by the presence of diverse bacterial species and host immune cells—the latter exhibiting specific responses to biofilm-associated bacteria. Bjarnsholt proposed that host transcriptome profiling to these different modes of infection can be used for diagnostic purposes.

Emphasizing the role of the host immune system in medical device infections, Phil Stewart (Montana State University, Bozeman, MT, USA) explained that a functioning host immune system is critical for the clearance of biofilm infections. Medical implants create a focus for infection because they are not functionalized by the host immune system and do not communicate effectively with immune surveillance functions. Using *S. aureus* as a model, it was found that these bacteria in normal human serum are readily cleared by neutrophils after attachment to a glass surface, whereas neutrophil attachment and subsequent motility were impaired in serum with decreased complement protein C3. The results indicate that sorbed host proteins are critical for effective neutrophil function on an implant surface, suggesting avenues for future immunotherapeutic approaches to prevent medical device infections.

Patients with long-term catheterization are quite prone to recalcitrant biofilm infections. The pathogenic yeast *C. albicans* is well recognized for candidiasis on catheters. Invited speaker Priya Uppuluri (Lundquist Institute, University of California, Los Angeles, Los Angeles, CA, USA) explained that there is missing knowledge on the host immune response to catheter-related candidiasis, and this is an important reason for our failure in controlling infections that disseminate from the original catheter to other areas of the body (67). She further demonstrated that abrogating the process of biofilm dispersal by inhibiting hyphae production from lateral yeast will help curb disseminated candidiasis. Studies on the role of newly discovered genes involved in anti-biofilm activity, as well as new host signaling pathways that target biofilms, are an ongoing focus.

Quorum sensing of infective populations can play multiple roles during host association of *P. aeruginosa*. Paula Giraldo Osorno (University of Gothenburg, Göteborg, Sweden) presented data showing that treatment of *P. aeruginosa* infections with the quorum-sensing inhibitor sodium salicylate (NaSa) reduced virulence factors while enhancing the immune response. Bacterial virulence was significantly decreased, while immune cell migration and bacterial clearance were promoted. Moreover, NaSa treatment attenuated secretion of pro-inflammatory cytokines *in vitro* and in a rat implant model. In the future, novel anti-quorum-sensing strategies may make an important contribution to the treatment of implant-related infections.

Diarrheagenic and uropathogenic *E. coli* strains utilize autotransporter proteins to switch from biofilm growth to invasion of the intestinal epithelium. J. J. Paxman (La Trobe Institute for Molecular Science, La Trobe University, Melbourne, Australia) solved the crystal structures of six self-associating autotransporter adhesins from several different bacteria. These adhesins are critical for bacterial cell-to-cell interaction and although they share structural similarities, differences in their modes of self-association influence the kinetics of aggregation and thus biofilm formation (68). Glycosylation of the adhesin TibA is further used to switch the function of this protein from promoting biofilms to invasion of the intestinal epithelium. Overall, the autotransporters showcase the adaptations bacteria have developed to modulate their levels of aggregation and biofilm formation.

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CONTROL, PREVENTION, AND ELIMINATION OF BIOFILMS

The ability to control, prevent, and eradicate biofilms is arguably one of the most compelling and challenging aspects of biofilm research. In this session, two invited and two selected talks were presented, providing different strategies spanning from natural plant-produced agents, changes in surface topography, and the use of nanobubbles and catalytic nanorobotics. Invited speaker Dacheng Ren (Syracuse University, Syracuse, NY, USA) presented his lab's work on the engineering of passive and active surface topographies to control biofilm formation. Initially, by investigating how bacteria interact with micron-scale topographies, Ren and colleagues highlighted a set of principles that guide rational design of antifouling topographies and experimentally validated these designs (69). Surfaces fabricated with grooves in various widths (5–20 μm) lead to spatial arrangements of colonizing bacteria in accordance with groove width and can be refractory to biofilm formation. Although these surface patterning approaches can prevent biofilm formation, they do not remove established biofilms. To address this limitation, surfaces with dynamic topography driven by magnetic action of micro size pillars were developed for more effective biofilm control. The active topography exhibited effective biofilm prevention and disruption of mature biofilms of several microbial species, making them more sensitive to antibiotic treatment. Ren described a prototype catheter with active surface topography that remained uncolonized for more than 30 days under the constant challenge of artificial urine with inoculated UPEC, in contrast to control catheters, which were completely blocked within the first week. Taken together, the results demonstrate the potential use of this strategy for biofilm-resistant materials.

In parallel to engineering surfaces for biofilm resistance, there have also been remarkable innovations with catalytic nanomaterials for removing biofilms from hard-to-reach surfaces. Invited speaker Hyun (Michel) Koo (University of Pennsylvania, Philadelphia, PA, USA) described his group's development of biocompatible iron oxide nanoparticles (NPs) that generate ROS formation and exert antimicrobial activity. The nanoparticles are also magnetic, and their movement, aggregation, and momentum can be controlled by applying an electromagnetic field. These catalytic antimicrobial robots can kill, degrade, and remove adherent biomass (70). By creating custom NPs by aggregation or 3D molding, Koo and colleagues were able to design specific shapes that can be precisely controlled by the magnetic field to "shovel" and remove the biofilm. Combined with the ROS catalytic action of the NPs, a combined kill and removal action is obtained. A modified approach utilized the dynamic spatial organization of the NPs to form bristle-shaped NP aggregates that can rapidly adjust their conformation, length, and stiffness to apply high-shear stress. These properties provide the required flexibility to treat surfaces with complex topography, enabling access to narrow grooves and cavities (71). The bristle technology was applied to human teeth that represent complex three-dimensional geometries, and impressive biofilm inactivation and removal at microscale precision were demonstrated. Taken together, these "kill-degrade-and-remove" catalytic antimicrobial robotic systems hold great potential for fighting biofilm infections.

Another physical approach to biofilm control employs the use of nanobubbles (NBs), concentrated bubbles smaller than 1 μm, which can be created by pumping water through a special Turbu-Flow device under high pressure. NBs are fairly stable and have a relatively large surface area that promotes chemical reactions (72). When the NBs collapse, they create ROS and have antimicrobial activity. Thu Le (laboratory of Chuanwu Xi, University of Michigan, Ann Arbor, MI, USA) described how treatment of 2-day-old *E. coli* flow cell biofilms with NBs for 5 h caused a 2-log decrease in biofilm cells and also a reduction in the planktonic bacterial load. Testing NB treatment on the more medically relevant biofilms of *E. faecalis,* a common root canal pathogen, proved to be more challenging, perhaps due to the complex environment. In this case, irrigation with NBs did not affect the overall bacterial load, but the combined treatment of NBs and

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sodium hypochlorite (NaOCl) resulted in a greater decrease than the chemical treatment alone, suggesting the possible efficacy of combined approaches.

Natural products can also have potent anti-biofilm activities. Mark Gomelsky (University of Wyoming, Laramie, WY, USA) reported on the identification of plant-based compounds that can inhibit biofilm formation by *Listeria monocytogenes*, a major food-borne pathogen, and cause of listeriosis. Biofilm formation on plant surfaces, including the surfaces of fruit and vegetables, is significantly enhanced by the ability of the bacterium to produce the Pss exopolysaccharide (73). Postdoctoral fellows Alex Fulano and Ahmed Elbakush in the Gomelsky lab examined biofilm formation and dispersion of *L. monocytogenes* wild-type, hyper-producing, and non-producing EPS strains on wood coupons and fresh produce pieces. The hyper-producing EPS strain readily colonized these materials. Surprisingly, with longer incubation, the biofilm on coupons derived from maple, hickory, and star jasmine detached and dispersed, in contrast to those on oak, birch, and apple, suggesting the presence of an antifouling compound. Nortrachelogenin-8′-O-β-D-glucopyranoside, a lignan present in aqueous maple extracts, was identified as a likely candidate that inhibits exopolysaccharide biofilms. Potent antibiofilm activity was also found in maple syrup. Given its status as a common foodstuff, maple syrup is generally regarded as safe and has significant potential in protecting fresh produce from *L. monocytogenes* biofilm-associated colonization.

INNOVATIVE APPROACHES AND NEW TECHNOLOGIES IN BIOFILM RESEARCH

The 2022 Biofilms Conference was notable with respect to the broad range of approaches brought to bear on the analysis and manipulation of biofilms. This session was specifically focused on new angles on analysis of biofilms. Invited speaker Herman Sintim (Purdue University, West Lafayette, IN, USA) described the rational design of drugs based on targets that are essential components of the Gram-positive bacterial cell with the goal of dispersing or preventing the formation of antimicrobial resistant biofilms. The first molecular scaffold targets cyclic dinucleotide cyclase signaling, specifically, cyclic di-adenylate monophosphate (c-di-AMP). In Gram-positive bacteria, the intracellular second messenger c-di-AMP regulates a myriad of physiological processes including biofilm formation, with low c-di-AMP levels coinciding with reduced biofilm formation but increased susceptibility to oxacillin. The c-di-AMP synthase (DAC) is a suitable target for signaling inhibition. Sintim's group identified several DAC inhibitors including hydroxybenzylidene-indolinones and GW5974 (74). These two compounds differ from previous DAC inhibitors in that they are cell permeable, possess antibacterial activities, and are capable of inhibiting biofilm formation. Gram-positive bacteria are also reliant on lipoteichoic acid (LTA) biosynthesis, as LTAs play pivotal roles in growth, DNA replication, and biofilm formation, making LTA inhibition a promising target (75). An inhibitor of the first step of LTA biosynthesis (HSG-95) developed by the Sintim lab proved to be a potent inhibitor of both staphylococci clinical isolates and methicillin resistant *S. aureus* biofilms formed *in vitro* and *in vivo* using a skin model of biofilm formation. Similar to LTA, targeting the membrane of non-growing bacteria has proven to be an effective antibacterial target, considering that compounds capable of killing persister cells are mostly membrane-acting compounds. An example is bithionol, but this compound is toxic. Sintim and colleagues have developed potentially safer compounds containing SF5 or SCF3 moieties which are selective for bacterial membranes and fluidity.

Continuing with the chemical theme for biofilm analysis, invited speaker Jerry Troutman (University of North Carolina-Charlotte, Charlotte, NC, USA) described the development of tools for the detection of polysaccharides and surface polymers by the abundant mammalian gut symbiont *Bacteroides fragilis*, with the goal of reconstructing the capsular polysaccharide A (CPSA) biosynthesis pathway *in vitro*. CPSA is a four-sugar repeating unit polymer that plays an important role in mammalian immune system development and may have therapeutic potential in model systems for autoinflammatory and autoimmune diseases, including multiple sclerosis. The Troutman lab approach

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makes use of fluorescent polyisoprenoid chemical probes that are easily detectable anchors for key phosphoglycosyl and glycosyl transferases (76). Reconstruction of the synthesis of CPSA in *E. coli* revealed that the enzymatic assembly of the tetrasaccharide repeat unit of CPSA occurs in a sequential "single-pot" reaction and that the *B. fragilis* CPSA biosynthesis gene locus alone is not sufficient for CPSA production. Instead, additional sugar-modifying enzyme encoding genes from *Campylobacter jejuni* and *Vibrio vulnificus* were required to complete the development of an effective CPSA assembly system in *E. coli*.

Powerful microscopic and image analysis approaches often drive major advances in biofilm research. An excellent example was provided by Jing Yan (Yale University, New Haven, CT, USA), who described work from his group exploring the biophysical mechanisms underlying the development of biofilms grown in confined agarose gels. Using single-cell live imaging and mutagenesis of *Vibrio cholerae*, Yan demonstrated that the morphodynamics and cell ordering in embedded biofilms are fundamentally different from those of the biofilms on flat surfaces (77) (Fig. 5). Moreover, rheological measurement indicated that stiffness of the environment and the biofilm itself, jointly with cell-to-surface adhesion determined biofilm shape, growth dynamics, cell fate, and internal architecture. The findings are potentially applicable to biofilms embedded in mucus or host tissues during infection and provide insight into how bacterial biofilms compromise between their inherent developmental program and the mechanical constraints imposed by their micro-environment.

To explore the general assembly rules governing biofilm formation, Shawna Pratt (laboratories of Connie Chang and Matthew Fields, Montana State University, Boseman, MT, USA) described efforts underway in the Fields lab to develop 3D bioprinting methods with laser lithography as a rapid fabrication technique to provide control over the structure and composition of living materials with thicknesses between 100 and 300 μm. Hydrogels with either an encapsulated bacterium (*Pseudomonas fluorescens*) or green alga (*Chlorella*) were 3D printed, and growth was monitored spatially and temporally in the hydrogel matrix based upon red-fluorescent protein in *P. fluorescens* or chlorophyll for *Chlorella*. The 3D printing process had little effect on viability but enabled the growth of biofilms as aggregates, with aggregate size depending on the location within the hydrogel. The 3D-printing approach provides great control of aggregate distribution as

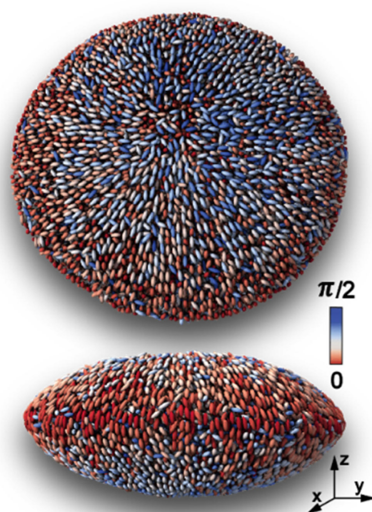

FIG 5 Single-cell 3D reconstruction of an embedded *Vibrio cholerae* biofilm grown in a 2% agarose gel (top and side view). Cells are color coded according to their angles with respect to the short axis of the oblate-shaped biofilm. (Image courtesy of Jing Yan, reproduced with permission.)

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well as density and is certain to yield new insights into the impact of starting conditions on eventual biofilm structure.

The second of the Early Career Symposium awardees for best presentation was included in this final session (Table 3). Ian Reynolds (laboratory of Clay Fuqua, Indiana University, Bloomington, IN, USA) described his studies of the unipolar polysaccharide adhesin UPP of *A. tumefaciens*. The UPP is composed of two distinct polysaccharide species, distinguished by the presence of *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) residues in addition to other monosaccharides (78). Each of these polysaccharides requires a specific Wzy-type polymerase, designated UppW and UppY, respectively. A genetic screen for additional pathway-specific mutants identified *rfbA* and *rfbD*, predicted to encode a glucose-1-phosphate thymidylyltransferase and a dTDP-4-dehydrorhamnose reductase. The *rfbA* and *rfbD* genes are located in a four-gene operon predicted to be involved in the synthesis of the dTDP-L-rhamnose, a frequent component of the LPS core or O-antigen. In addition to decreasing production of the GalNAc-containing UPP species, mutants for *rfbD* and *rfbA* aggregate non-specifically and also manifest deficiencies with flagellar motility, although they are fully flagellated. These results suggest a connection between LPS, at least one of the UPP species, and flagellar motility.

KEYNOTE PRESENTATION: ROBIN PATEL

The conference closed with a compelling talk by recent past ASM president Robin Patel (Mayo Clinic, Rochester, MN, USA), who framed the problem of periprosthetic joint infections (PJIs) in a historical context. Extremely rare in the 1990s, the occurrence of infections associated with joint replacements has substantially increased with the increased occurrence of those procedures, with the numbers of infections expected to reach into the millions in the coming years (79). Thus, despite years of work investigating underlying causes and mechanisms, PJIs remain a major clinical problem driven by the twin challenges of diagnosing and treating them. In a significant percentage of cases, the causative agent cannot be isolated or identified, and in up to one-third of the cases, infections derive from polymicrobial sources. The Patel lab works on improving diagnostics and therapeutics, efforts aided by clinical trials and their investigations into the responses of the host and bacteria to the infection. The group capitalizes on advances in technology and methodology in the microbial sciences to address the continuing problem, including the application of shotgun and then targeted metagenomics, as well as proteomics and transcriptomic analyses. This work has enabled the evaluation of tests for PJIs that include assaying for leukocyte esterase, C-reactive protein, and alpha-defensin. These tests are associated with neutrophil-associated responses, as these host-defense cells are a key part of the response to PJIs. Finally, Patel described some recent work exploring new treatments, including the use of alternative rifamycin drugs such as rifabutin and rifapentine that worked well in combination therapies with vancomycin, which are now being evaluated in the context of patients, and novel therapeutics such as the use of lysins. While much work remains to be done, the forward-looking, multi-disciplinary approaches espoused by the Patel group provide hope that the problem of PJIs may be more readily addressed in the future.

SUMMARY AND PERSPECTIVES

Analogous to the emergent properties of microbial biofilms, the community of biofilm researchers benefits and synergizes in the context of an in-person conference such as that provided by the ninth ASM Conference on Biofilms. With a gap of 4 years between the eighth and ninth iterations of the conference and the intervening destructive impact of the COVID-19 pandemic on research and researchers alike, our ability to once again convene our community was rightfully treated as a cause for celebration. Indeed, coming through on the other end of the pandemic to hear about fantastic research on experimental advances in biofilm research and engage in person-to-person dialogs about the microbial sciences were renewing and rewarding. Moreover, this

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conference incorporated numerous special features that enhanced the overall experience. In particular, it successfully strove to highlight the contributions of early career scientists through a separate symposium organized by early career scientists and by selection of their abstracts for presentation in both short and lightning talk formats. The resulting high energy and positive tone of the conference were undeniable. With this level of enthusiasm, with the dynamic research activity of the community, and with the threat and potential posed by biofilms, it seems clear that we can rest assured of a productive future for the field of biofilm research.

The conference ended with the announcement that ASM will support a tenth ASM Conference on Biofilms scheduled for 2025, and co-chaired by Clay Fuqua and Karin Sauer.

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