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REVIEW

Options and Limitations in Clinical Investigation of Bacterial Biofilms

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SUMMARY Bacteria can form single- and multispecies biofilms exhibiting diverse features based upon the microbial composition of their community and microenvironment. The study of bacterial biofilm development has received great interest in the past 20 years and is motivated by the elegant complexity characteristic of these

multicellular communities and their role in infectious diseases. Biofilms can thrive on virtually any surface and can be beneficial or detrimental based upon the community's interplay and the surface. Advances in the understanding of structural and functional variations and the roles that biofilms play in disease and host-pathogen interactions have been addressed through comprehensive literature searches. In this review article, a synopsis of the methodological landscape of biofilm analysis is provided, including an evaluation of the current trends in methodological research. We deem this worthwhile because a keyword-oriented bibliographical search reveals that less than 5% of the biofilm literature is devoted to methodology. In this report, we (i) summarize current methodologies for biofilm characterization, monitoring, and quantification; (ii) discuss advances in the discovery of effective imaging and sensing tools and modalities; (iii) provide an overview of tailored animal models that assess features of biofilm infections; and (iv) make recommendations defining the most appropriate methodological tools for clinical settings.

KEYWORDS animal host models, biofilms, flow cells, imaging, quantification

INTRODUCTION

Biofilms are multidimensional communities in which resident bacteria coexist within the self-derived extracellular matrix (ECM) (1, 2). Although the developmental stages leading to biofilm formation appear to be conserved (Fig. 1), every species (or consortium of species in the case of polymicrobial biofilms) forms a unique multicellular community (3, 4). This protected network possesses the ability to evade environmental threats, such as antimicrobials and host defense mechanisms (5).

Biofilms account for 80% of chronic microbial human infections, leading to increased rates of hospitalization, elevated health care costs, and increased mortality and morbidity rates (6). Upper and lower respiratory tract diseases, native valve endocarditis, chronic otitis media, eye infections, chronic wounds, diabetic foot ulcers, urinary tract infections (UTIs), and periodontitis are all biofilm-associated diseases (7–12). Biofilms can also develop on abiotic surfaces, including medical devices such as orthopedic prostheses, artificial cardiac valves, coronary stents, intravascular and urinary catheters, neurosurgical, cochlear, and breast implants, dentures, and ventricular-assist and ocular devices (13).

Since the term "biofilm" was introduced in 1978, the intrigue and excitement surrounding a new microbiological field of study have produced opportunities of environmental, industrial, and clinical importance (14). Biofilms made a global impact in the literature, and their study continues to generate more questions. Because of the mechanical, physicochemical, microbiological, and medical components of biofilms, different disciplines views biofilms from different perspectives: chemists focus on organized chemicals, physicists deal with thermodynamics, and biologists examine the microbial physiology that affects biofilm formation and also unravel resistance patterns, yet all are left with the question of how all these components comprise the biofilm-associated threat. The unique nature of biofilm communities within the context of infection makes the constant development of new strategies acting against biofilms, as well as architectural and behavioral investigations, an evolving necessity (15). The last decade has seen a multitude of diverse methodological tools and enabled a context for comparative use and analysis that follows the rationale that biofilms thrive on living and inanimate surfaces by adaption and survival. Their versatile behavior leads to extended (by design) experimental diversity and subsequently confounds efforts to evaluate biofilm-specific *in vitro* antimicrobial susceptibility. For the methodological piece alone, it should be noted that the risk of erroneous data acquisition is significant and that limited accuracy and reproducibility of viability assays have been recorded (16, 17). This description fails to capture the inconvenient reality. Dye aggregates will not bind stoichiometrically to complex communities (18). Microscopic enumerations, by default, provide indirect population assessments. Conventional colony formation assays

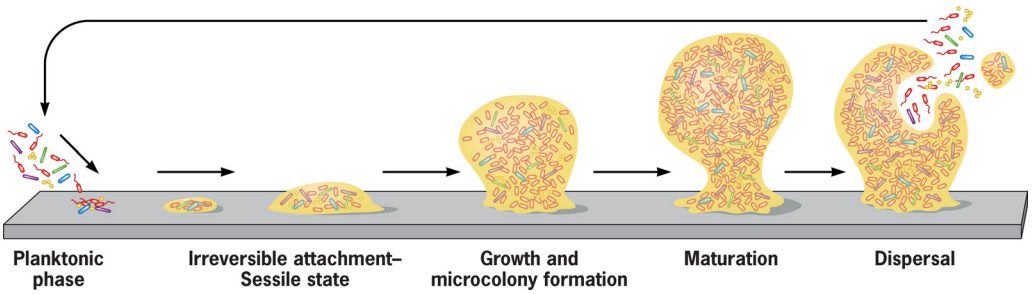

FIG 1 Developmental stages in biofilm formation. One or more planktonic bacterial species adhere to a biotic/abiotic surface. Attached bacteria grow as a multicellular community, forming microcolonies in which they multiply and mature. This microbial infrastructure results in the development of a mature biofilm. Eventually, biofilms serve as bacterial reservoirs that are transmitted back to the environment through biofilm dispersal and then colonize new surfaces. (The concept of this figure was inspired by reference 71.)

have limited value, as the attempt to "normalize" these communities in test tubes often requires processes that disturb the members.

In this review, we (i) emphasize classical approaches for biofilm monitoring and quantification, (ii) highlight the evolution of imaging tools for architectural analysis of biofilm communities, and (iii) provide examples of methodological applications, including apparatuses to trace clinical biofilms. Despite the wealth of research approaches, there is an unmet need to filter the most interesting contribution per methodological group. We present here an understanding of the advantages and disadvantages of each group to help guide current research in this field of study.

MULTIDIMENSIONAL ARCHITECTURE OF BIOFILMS

The ECM typically includes some type(s) of polysaccharides, proteins, and/or DNA (19). However, the ECM structure differentiates according to (i) the species or strains comprising the biofilm (20, 21); (ii) the conditions during development, and in turn the expression of bacterial factors (22); and (iii) the spatial location sampled within any given biofilm (23, 24). The ECM components affect structure, physiology, interactions with the surrounding environment, resistance toward antibiotics, and host defense mechanisms (25–28).

Biofilm architecture is highly variable. Bacteria can build exposed or submerged biofilms on either biotic or abiotic surfaces and under static or shear-flow conditions or, alternatively, coalesce directly in the host, as seen in intracellular bacterial communities (IBCs) involving uropathogenic *Escherichia coli* (UPEC) and *Klebsiella pneumoniae* (29–33). Another example of complex biofilm architecture is that of sputum-encased endobronchial *Pseudomonas aeruginosa* biofilms, which form aggregated clusters of bacterial cells surrounded by polymorphonuclear leukocytes (PMNs), the PMN-released enzymes elastase and collagenase, and oxygen radicals in the cystic fibrosis (CF) lung (34).

Differences among biofilm communities are also created in response to incoming signals and have importance in bacterial dispersal triggered by nutrient availability modifications (35). D-Amino acids, for example, have been reported to act individually or synergistically to trigger disassembly of biofilms and to inhibit pellicle formation by *Bacillus subtilis* and other species (36). The process happens through D-amino acid internalization in the bacterial cell wall affecting the anchoring of the amyloid-like protein TasA (37). Likewise, the cyclic diguanylate monophosphate (c-di-GMP) concentration affects matrix and structural component production, motility, cell attachment, and eventually biofilm formation in a number of species (38–41).

Surface-Associated Biofilms and Importance of the Substratum

Surface-attached biofilms forming colonies are valuable for studying bacterial community architecture on a solid surface (Fig. 2A). Interestingly, different gene expression patterns are observed by comparing biofilms attached to solid surfaces and planktonic

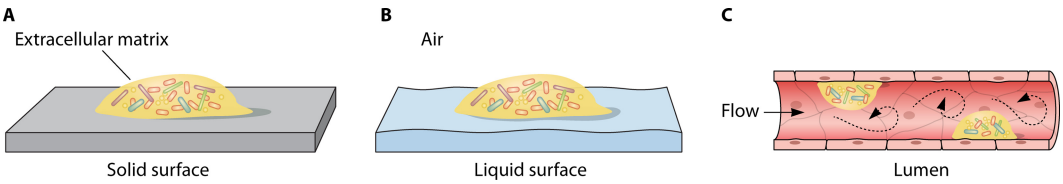

FIG 2 Biofilm types. (A) Surface-attached biofilms form colonies on a solid surface and are highly dependent on the substratum material. (B) Pellicles are formed in the air-liquid interface of fluids in nature or in the lab. Cells are bound together, forming a distinct macroscopic floating infrastructure. Thick pellicle formation requires the presence of exopolysaccharides (EPS). (C) Submerged biofilms develop under flow conditions. Biofilm formation under flow conditions is achieved in either indwelling catheters or suitably adapted lab devices.

bacteria grown in liquid cultures (42–47). Surface-associated biofilms are highly dependent on the substratum material and may or may not be exposed to air. Among the most common materials that promote biofilm formation on abiotic substrata are polyvinyl chloride (PVC), silicone, polystyrene, and metal (48–50). In microbial keratitis, for example, *P. aeruginosa* exhibits preferential adhesion to polymeric contact lenses (51), while in urinary catheters bacteria are preferably adherent to silicone and PVC biomaterials (52). As far as air exposure is concerned, the acquisition of antimicrobial resistance (AMR) and tolerance to xenobiotics has been attributed to (i) adequate oxygenation required for bacterial metabolism and (ii) facilitated DNA exchange due to the juxtaposed distribution of the bacterial cells in the well-organized biofilm cluster at the air-liquid interface (53).

Apart from surface composition, surface coatings can also play a role in biofilm formation. Blood components (fibrin, laminin, collagen, fibronectin, and immunoglobulins) compose the fibrin sheath that fosters the adherent growth mode around and into the air-deprived lumen of central venous catheters (CVC). Although results from both *in vitro* and *in vivo* studies regarding fibrin sheath-coated surfaces are inconsistent, reports of enhanced incidence of persistent bacteremia confirm the biofilm formation attributed to fibrin coating for some species (54).

Biofilms that take up nutrients directly from the surface to which they adhere (such as bacterial colonies on agar plates) are highly dependent on the substratum material (55, 56). In this case, the community grows outwards from the center of the colony, and architectural complexity most likely increases in response to nutrient and other environmental gradients that are created over time (45, 55, 56). Among these biofilms are the type called pellicles, which have been described as floating biofilms formed on a liquid surface (Fig. 2B) (42). Examples of bacteria that form liquid surface-associated pellicles include different strains of *E. coli* and *Salmonella* spp. (23, 57–59). This type of biofilm exhibits various degrees of meniscus growth, strength, and structure and possesses matrix-embedding, host-derived extracellular polymeric substance (EPS) components, laying the foundation for involvement in clinically relevant conditions (29, 60, 61). The liquid substratum provides the primary source of nutrients for the growing community. The term "pellicle" has also been used for dental diseases caused by biofilms formed from multicellular aggregates that require saliva proteins for attachment (62, 63).

Submerged Biofilms

In addition to surface-associated biofilms, bacteria can form submerged biofilms under both static and shear-flow conditions. These types of biofilms are perhaps the most relevant in most chronic infectious disease states, as most of the device-associated infections involve the formation of submerged biofilms. Pathogenic biofilms formed on all types of catheters, including urinary and central/peripheral venous access lines, endotracheal and nasogastric tubes, and cerebrospinal fluid shunts, as well as on artificial cardiac valves, are examples of multicellular communities developing under variable-flow conditions (Fig. 2C) (64, 65). In catheters of all types, bacteria are introduced from the outside environment into the catheter lumen during catheter insertion

and can swim or are carried by normal fluid flow. Bacteria then attach to the abiotic material by using adhesive fibers. Host biomolecules, such as fibrinogen, laminin, collagen, and fibronectin, also serve as platforms for bacterial adherence, as they become deposited on the surface in both central venous and urinary catheters shortly after insertion of the device (62, 66). Bacterial expansion upon adherence leads to the formation of the biofilm community, a process known as maturation.

Laminar/semilaminar flow (implicated in biofilm formation in blood vessels), turbulent fluid mechanics (characterizing prosthetic valves), and discontinuous trickle (referring to catheter-associated UTIs [CAUTIs]) are several examples of fluid flow dynamics (67–69). For each condition, suitably adapted lab devices that contain simultaneous growth medium supply and waste removal are developed. These flow systems create optimal conditions for the generation of mature biofilms. Culture preparation, surface conditioning, and adjusted methods provide lab substrates mimicking clinical conditions. A characteristic example involves the evaluation of four CVC *Staphylococcus epidermidis* biofilm infection models that differ in material type (glass versus polymer) and nutrient presentation (static versus continuous flow) (70).

Roots of Biofilm Phenotypic Resistance

The biofilm life cycle ends with the dispersal of bacterial cells stemming from the biomass after maturation (71). The dissemination and colonization of new target sites explain recalcitrant chronic infections within the host; biofilm-originating cells form bacterial niches with resistance phenotypes at the newly colonized sites. The biofilm dispersal process is controlled by environmental signals (oxygen, nutrients, temperature, and signaling molecules), intracellular reduction of the concentration of c-di-GMP, and upregulation of motility or quorum sensing (QS) genes, though many bacterial dispersal signals remain cryptic (13, 72, 73).

A single clonal population alters the growth rate and turns on adaptive pathways due to multiple environmental signals. The study of this complex yet distinct adaptation process elucidates changes in chronicity that contribute to fitness without being limited to specialized cells and signals (74). Since the environmental gradients surrounding the microbial community influence biofilm composition, phenotypically distinct subpopulations arise, including extracellular matrix producers, adhesive fiber producers, motile bacteria, and metabolically quiescent and/or antibiotic-tolerant bacteria (23, 24, 75, 76). Persisters emerge through diverse cellular and molecular phenomena, including biofilm matrix protection against the host immune system, redundant toxin-antitoxin (TA) modules induced by DNA damage (SOS response), nutrient elimination, the age of the inoculum, and the downregulation of genes related to motility, cell division, and protein synthesis (77–79). The quiescent phenotype is actively present in chronic and recalcitrant infections surviving under antibiotic pressure. For example, the reversible overexpression of the HipA and RelE toxins has been implicated in the interruption of cellular functional processes in *E. coli* under stress. Hence, persisters prevail without multiplying or being killed, exhibiting tolerance but not resistance, and therefore do not qualify as mutants (80, 81). The slow-growing small-colony variant (SCV) subpopulation is another equally puzzling phenotype that complicates biofilm formation as well as treatment and diagnosis of biofilm-related disease. The contribution of SCVs to biofilm formation has been studied for a rather small number of bacterial pathogens despite the huge clinical significance of persistent infections evading detection and complicating treatment (82, 83). In combination, these different phenotypes within microbial communities give rise to an extremely resilient community that can withstand many stressors and shield the resident bacteria from eradication.

Adaptive and phenotypic biofilm variations generate hurdles for the overall investigation and understanding of infection mechanisms. The partitioning of one or many different bacterial species is an additional unknown in this equation. Multispecies populations generate dynamic consortia with unique interactions [pathogen(s)-pathogen(s), pathogen(s)-commensal, and pathogen(s)-host], metabolic requirements,

and phenotypes, setting investigative limitations with respect to the *in vivo* clinical setup, infection pathogenicity, and response to treatment; on the other hand, monospecies biofilms constitute a minute fraction of both acute and chronic infections (84). Inter- and intraspecies interactions based on species-specific virulence traits set the stage for a complex microenvironment in which bacteria can exchange genetic markers and compete or cooperate for resources. The interplay among multiple pathogenic species enhances horizontal gene transfer of clinically prevailing phenotypic resistance elements. A fundamental trait of pluralism in biofilm composition is based upon the presence and content of nonbacterial elements. Examples of bacterial-fungal cooperation within the protected environment of a biofilm include that in wounds, the oral cavity, and urinary tract infections. This evolutionary tactic provides a stable survival equilibrium among kingdoms (85, 86). Therefore, explaining the rationale for the clinical relevance of multispecies biofilms is not trivial. While multispecies biofilms are the most clinically relevant infections, their study has been the most limited due to the complexity of each community, the lack of knowledge regarding the identity and abundance of each biofilm resident, and the technical limitations associated with different biofilm setups. Examples include *Haemophilus influenzae* competing with *Streptococcus pneumoniae*, but not with *Moraxella catarrhalis*, in ear infections; *Staphylococcus aureus* and *P. aeruginosa* cooperating for survival in chronic wound infections; group B streptococci promoting survival of UPEC in UTIs; and *Porphyromonas gingivalis* and *Treponema denticola* exhibiting synergy in biofilm formation involved in periodontal disease (87–92).

Since the onset of the first biofilm studies, several technological advancements have greatly enhanced our ability to molecularly dissect biofilm architecture and characteristics both temporally and spatially. Below is a comprehensive review of the methodological landscape used for the study of biofilms, presenting a blend of classical and more recent technologies.

LABORATORY SETUPS

Cutting-edge points regarding technical information are discussed and the strengths and limitations of the most popular laboratory devices are provided in Table 1. All biofilm species are not created equal, so they often require specific experimental conditions matching the developed methodological tools. Some tools are more practical and relevant than others, covering adherence, life in a "turbulent flow habitat," or the effect of coaggregation and flagellation on all types of bacteria.

Culturing Biofilms under Static Conditions

The complexity of colony biofilms on agar plates was overlooked for decades due to the lack of advanced methodologies to probe architecture differences (Fig. 3A). This method generates a stable structure and limits the possibility of cell detachment, ensuring that observed differences in cell numbers are due to cell death rather than detachment (93).

Microtiter plate-based assays were first described in 1985 for assessing staphylococcal adherence to plastic tissue culture plates (94) (Fig. 3B). Bacterial inocula with standardized concentrations are placed in a 96-well microtiter plate made of PVC, polystyrene, or other material and incubated aerobically at 37°C for designated time frames (1 to 4 h for initial attachment and ~20 h for biofilm formation, depending on the species). Planktonic cells are removed by gentle washing, and the adherent cells are stained with crystal violet (CV) and quantified by spectrophotometry (95, 96). Studies concerning biofilm-forming isolates have exhibited that motile bacteria, including flagellated *E. coli*, *P. aeruginosa*, *Vibrio cholerae*, and *Salmonella enterica*, tend to coaggregate at the air-liquid interface. In contrast, the nonmotile cocci, including enterococci (with the exception of the motile organisms *Enterococcus casseliflavus* and *Enterococcus gallinarum*) and staphylococci, form aggregates at the microtiter plate base (97–102). Microtiter plates with removable silicone or polystyrene disks at the

TABLE 1 Assays and lab devices used for biofilm formation evaluation

Assay or device	Advantage(s)	Disadvantage(s)	Reference(s)
Static conditions			
Agar plating	Stable structure	Difficulties in handling colonies due to increased growth rate	93, 435
	Low possibility of cell detachment	Variations in bacterial strain motility rate	
Microtiter plate assay	Simple	Difficulties in mature biofilm generation due to lack of nutrient supply	436, 437
	Rapid	Poor attachment to abiotic surfaces by several clinical strains	
	Highly reproducible	Limited substratum options	
	Antimicrobial susceptibility assay	Inability to test biofilms with flow viability validation	
Air-liquid assay	Simple	Visualization of early-stage biofilms only	93, 95
	Direct microscopy	Planktonic cells may hinder biofilm observation	
	Coverslips can be used in the absence of an inverted microscope	Washing and staining steps required	
	Stained coverslips do not require immediate microscopy (can wait ~1 wk)		
BRT	Accurate, automated, reproducible, and rapid	No biofilm viability validation	105, 108
	Amenable for high-throughput screening	Specified software and biofilm index provide the result	
	No need for staining or washing steps	Inability to test biofilms under flow	
Flow conditions			
Kadouri system	Large biomass generation	Planktonic cells may not be discarded due to low flow	93
	Applies to various growth demands	Wells overflow	
	DNA microarray assay facilitation	Close monitoring required to avoid waste tube blocking and drying of the wells	
	Proteomics facilitation		
	Mature biofilm formation		
Drip-flow cell reactor	Large biomass produced	Limitations in confocal microscopy visualization	95, 126
	Viable cell enumeration	No nutrient laminar flow	
	Medical material surface antibiofilm testing		
	Molecular study facilitation		
	Antimicrobial susceptibility assay facilitation		
	Microsensor monitoring study		
Robbins device	Structural integrity conserved	Limited *in situ* biofilm visualization	119, 438
Modified Robbins device	Study of biofilm physiology	Biofilm destruction during sampling for quantitative analysis	
	Biofilm-related bacterial metabolic product investigation		
	Investigation of immune response to biofilm-associated bacteria		
	Allows aseptic sampling and plug handling		
Rotating-disk reactor	Surface material-associated study	Limitations in confocal microscopy visualization	126, 127, 128, 439
	Biofilm growth rate study	For antimicrobial compound testing, coupons need transfer into 96-well plates	
	Biomass structure investigation		
	Antimicrobial susceptibility testing		
	Identical biofilm production		
Calgary device	Antimicrobial susceptibility assay	Sonication may lead to erroneous results	436, 440
	3D imaging facilitates structural analysis	Time-consuming	
	Viability assay facilitation	Specific fluorophores required for CLSM	
		Sample destruction due to fixation required for microscopy	
Microfermentors	Genetic and biochemistry analysis facilitation		441
	Study of biofilm adherence and formation		
	Multi- or monospecies biofilm evaluation		
	Mature biofilm formation		

(Continued on next page)

TABLE 1 (Continued)

Assay or device	Advantage(s)	Disadvantage(s)	Reference(s)
Microfluidics-based device	Noninvasive technique Continuous biofilm formation assessment Enhanced physiological relevance of live-cell assays due to shear flow Simplicity of usage Host-bacterium interaction analysis Real-time visualization of biofilm growth	No metabolic products obtained (closed system)	

bottom can serve as platforms to facilitate microscopic and molecular examinations (97).

An air-liquid assay enables qualitative analysis of biofilm formation through direct phase-contrast microscopic visualization of the early-stage bacterial aggregates in flat-bottomed wells (Fig. 3C). Bacterial inocula placed in a 24-well flat-bottomed plate are incubated at an angle or coverslips are placed at an angle into wells for the appropriate incubation time; coverslips are washed and stained with crystal violet or with a fluorescent antibody against a species-specific antigen or a protein of choice. A conventional or fluorescence microscope can be used for biofilm visualization (93). The *sad* (surface attachment defective)-associated reversible and irreversible attachment of *P. aeruginosa* was investigated by this assay to decipher the pseudomonal developmental shift from the planktonic to the biofilm state in the CF lung (103). Biofilm formation on epithelial cells, assessed by the air-liquid method, served as an *in vitro* model of chronic rhinosinusitis due to *P. aeruginosa* PAO1 (104).

The BioFilm ring test (BRT) is a newly available tool for the study of biofilm formation kinetics that was developed in the last decade. It detects biofilm formation in modified 96-well polystyrene microtiter plates by use of magnetic microbeads and a scanning plate reader (105) (Fig. 3D). Biofilm-associated adherence is determined when beads remain scattered after the application of a magnetic field; in contrast, beads are immobilized in the center of the well bottom in the presence of planktonic cells (105, 106). Examples of BRT biofilm formation kinetics include those of (i) the nontypeable *H. influenzae* (NTHi) strains isolated from body fluids (blood, sputum, and pleural and cerebrospinal fluid) of individuals with nonbacteremic, community-acquired pneumonia and chronic obstructive pulmonary disease (COPD) and from middle ear fluid of patients with otitis media; (ii) the *S. aureus* and *S. epidermidis* strains from acute and chronic osteomyelitis and infectious arthritis cases; and (iii) the *P. aeruginosa* strains from CF patient sputum samples (107–110). A recent extension of BRT is Antibiofilmogram, which is used for susceptibility profile testing of bone and joint infection-related *S. aureus* biofilms with 11 widely used antibiotics. This method aims to facilitate therapeutic choices by narrowing the window of truly efficient antibiofilm treatment options (111).

Culturing Biofilms under Flow Conditions

Continuous-flow cultures enable the formation of mature biofilms in chambers covered with coverslips or on silicone or latex tubes fitted to a peristaltic or syringe pump. The peristaltic pump facilitates flow of fresh growth medium, whereas planktonic cells and waste are removed. The biofilm formed is monitored microscopically after introducing fluorescent proteins or reporter genes (112). Reporter genes are informative for the stage-specific physiological changes that occur under dynamic conditions and differentiate biofilms from their planktonic counterparts (21, 23, 113). Green fluorescent protein (GFP) serves as an example of the reporter signals associated with the *csgBA* genes, which have been used extensively in biofilm interventions for UPEC (23).

Among the advantages of continuous-flow models is the ability to compare the effects that different media, oxygen concentrations, temperature shifts, and substances exert on a biofilm at all developmental phases. These models also offer evaluation of

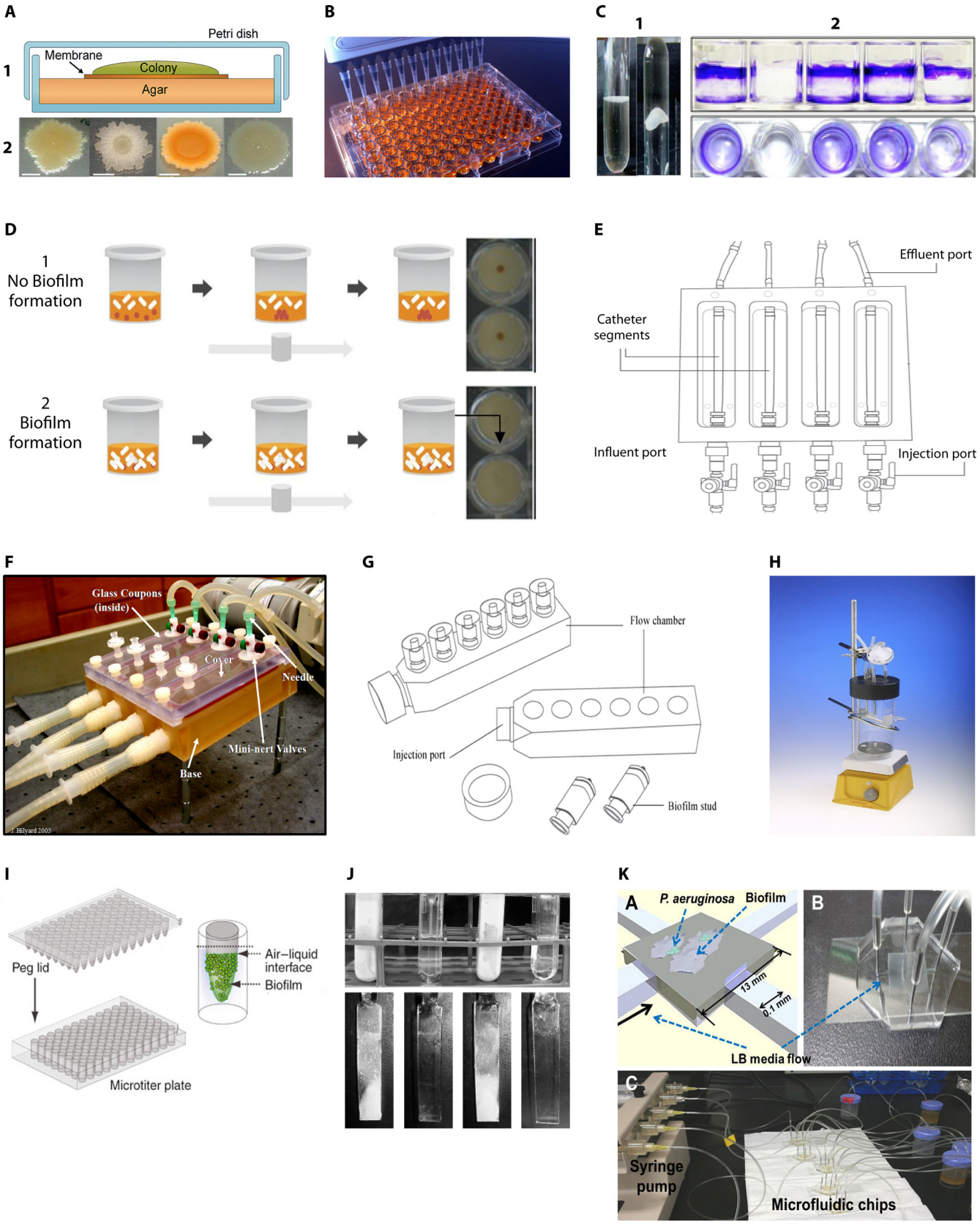

FIG 3 Laboratory setups. (A) Colony biofilms on agar plates. (1) Schematic diagram of a colony biofilm. (2) Various types of macrocolonies grown on agar medium. (Panel 1 reprinted from reference 427 with permission of the publisher; panel 2 reprinted from reference 428.) (B) Microtiter plate. (Photograph taken and kindly provided by Alex Hall.) (C) Air-liquid biofilms. (1) Pellicle formation at the air-liquid surface. (2) Crystal violet staining was performed to assess

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the effects that transiently occurring molecules, such as antibiotics or adherence inhibitors, have on biofilms. However, the technical disadvantages of continuous-flow biofilms include increased experimental complexity as well as possible formation/trapping of air bubbles in the setup tubing. This can perturb proper medium flow, affect the architecture, and put the system at risk for contamination (114, 115).

The Kadouri system is an intermediate system between static biofilms and low-flow cells (Fig. 3E). The major difference from static assays is that the wells are part of a closed system with two outputs, one for the continuous fresh medium supply via an adjusted pump and one for the removal of waste and planktonic cells, while the difference from flow-cell systems lies in the minimal shear forces (93, 116).

Drip-flow cell reactors enable biofilm formation in the air-liquid interface at low shear forces (117) (Fig. 3F). A biofilm is formed in channels (mainly silicon tubes) containing glass coupons or catheters, and a large biomass is produced (20, 22, 61). An *in vitro* model has been applied for bacteriophage-based bacterial biofilm inhibition on medical device surfaces. This model involved *S. epidermidis* biofilms on hydrogel-coated urinary and central venous catheters with a modified drip-flow biofilm reactor; the modification was a shift from drip to constant flow (118).

The Robbins device (RD) generates submerged biofilms growing in aqueous systems that can be used for the interrogation of multispecies communities (119, 120). Culturing multispecies biofilms *in vitro* can become complex regarding the microenvironment simulations. At present, multispecies biofilms are formed either by coculture of different preacquired clinical strains or by transfer of a mixed environmental population into the experimental setup (121). So far, the most commonly applied device is the modified Robbins device (MRD) (Fig. 3G), involving mono- or multispecies biofilm formation on catheter segments of various materials under flow conditions. Thus, MRD enables real-life biofilm simulation on catheter surfaces and is considered amenable for *in vivo* evaluation of implanted devices and catheters (14, 122, 123). This setup has been applied for biofilm therapeutic interventions, including pharmaceutical preparations, such as minocycline and EDTA (M-EDTA), as well as the maintenance of catheter lumen patency and bacteriophage therapy for biofilm-related infections in CF patients (124, 125).

Rotating-disk reactors, including the CDC biofilm reactor, facilitate growth under controlled moderate shear stress and continuous-medium-flow conditions (126) (Fig. 3H). The reactor consists of a disk that rotates over a magnetic base and coupons of various materials that promote biofilm growth. A glass reactor vessel enables medium supply and waste removal; coupon removal at desired intervals is feasible (126). An alternative method involves concentric reaction chambers with cylinders enabling biofilm formation. Modifications in speed or the cylinder's diameter affect the cell density and biofilm structure (127, 128). Rotating-disk reactors enable antimicrobial agent and antifouling material efficacy, and the removable disks serve as testing surfaces for antibiofilm compounds (21).

The peg lid Calgary biofilm device (CBD) generates biofilms on the bottoms of 96 pegs fitted in a polystyrene microplate lid or a multichannel tray containing a bacterial inoculum of a standardized optical density (Fig. 3I). Pegs are then sealed and incubated on a rocking table that creates shear forces at a specific temperature for a specific time. Planktonic cells are rinsed off, and biofilms are detached from the pegs by sonication and then placed into sterile 96-well microtiter plates with specific dilutions of the tested biocide to estimate the minimum biofilm eradication concentration (MBEC) after 24 h

FIG 3 Legend (Continued)

air-liquid biofilm formation on abiotic surfaces. (Panel 1 reprinted from reference 429; panel 2 reprinted from reference 430.) (D) BioFilm ring test. Photos of scanning microplates were taken with a plate reader after magnetization and show no biofilm formation (1) and biofilm formation (2). (Photos reprinted from reference 108.) (E) Kadouri biofilm system for flow biofilm study. (Reprinted from reference 431.) (F) Drip-flow reactor and various components. (Photograph kindly provided by the Center for Biofilm Engineering, MSU-Bozeman; see reference 117 for further details.) (G) Modified Robbins device. (Reprinted from reference 431.) (H) Rotating-disk reactor. (Photograph kindly provided by BioSurface Technologies Corp.) (I) Peg lid Calgary device. (Adapted from reference 432 with permission from Macmillan Publishers Ltd.) (J) Biofilm growth in microfermentors. (Reprinted from reference 433.) (K) Microfluidic device and experimental setup for biofilm formation (Reprinted from reference 434 with permission.)

of incubation (129, 130). The study of sputum biofilm-forming *P. aeruginosa* strains isolated from CF patients is one example of CBD being used to compare the efficacies of multiple antibiotic combinations (131, 132).

Microfermentors use slides of various materials as the substrate to form bacterial communities, while a constant medium flow and sterile air supply are provided (5, 133, 134) (Fig. 3J). The large mass of biofilm generated through this assay provides information not only about the biofilm-forming ability of the tested strains but also about the antimicrobial susceptibility profile (5, 134). Methicillin-resistant *S. aureus* (MRSA) strains appear to switch between proteinaceous and exopolysaccharidic biofilm matrices according to the provided substrate and environmental conditions in microfermentors (135).

Microfluidics-based devices, including the relatively recent BioFlux device, are fully integrated platforms consisting of modified 96-well plates with laminar flow chambers, a shear-flow control system, an imaging system, and advanced software for data collection and analysis (Fig. 3K). Microfluidic channels enable fresh medium movement due to pneumatic pressure at a controlled flow rate, rolling velocity, and time. Antimicrobial agents can be delivered to the flow biofilms from the inlet to the outlet well. In high-throughput (HT) flow-cell biofilm viability assays, viable cells are quantified by epifluorescence microscopy coupled with chemical or genetic color coding; high-quality images are attributed to coverslip glass at the bottom of the wells (136). Modifications involving protocol adaptations (temperature, medium type, and concentrations) and supplemental accessories (software packages and modules) extend microfluidic applications by facilitating complex assays and multiple experiments (137). Examples of the use of microfluidics-based devices adapted for coculturing eukaryotic cell lines with bacterial aggregates include the study of HeLa cells with enterohemorrhagic *E. coli* (EHEC) and commensal biofilms to demonstrate the developmental events in a gastrointestinal (GI) tract infection (138). Another example provided real-time monitoring of osteoblast adhesion and viability on the Ti alloy surfaces of orthopedic implants infected with *S. epidermidis* (139).

Most of the existing lab setups for monospecies or multispecies bacterial biofilm formation also serve for biofilm-mammalian cell coculture systems. Both static and flow coculture biofilm model systems have been applied to study biofilm formation on biotic surfaces as well as the responses to various therapeutic approaches (140). These models aim to reflect the infectious process in real time and offer the advantage of examining the host-pathogen interaction. Various culture systems have been employed in biofilm-mammalian cell coculture models, including (i) human airway epithelial cells (CFBE cells) and human bronchial epithelial cells (BEAS-2B) cocultured with *P. aeruginosa* (140–142); (ii) a human oral keratinocyte cell line cocultured with multispecies oral biofilms involving *P. gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus intermedius*, *Veillonella dispar*, *Actinomyces naeslundii*, and *Prevotella intermedia* (143, 144); (iii) intestinal epithelial cells cocultured with *E. coli* O157:H7 (145); and (iv) human osteoblasts cocultured with *Staphylococcus epidermidis* (146).

QUANTITATION AND VIABILITY ASSAYS

Static experimental conditions and a discontinuous nutrient supply are limiting factors in assessing mature biofilms in a lab setting (147). Monitoring the viable bacteria within a biofilm can be achieved through (i) cultivation, (ii) metabolic activity detection, and (iii) membrane integrity evaluation. Conventional CFU enumeration fails to generate reproducibly reliable results for most biofilm quantification due to the presence of cell aggregates hampering distinct colony development as well as individual-species resolution, in the case of multispecies biofilms. Furthermore, vortexing or sonication before plating may lead to sample structure destruction or air bubble entrapment and erroneous results due to partial cell detachment (2, 148).

The crystal violet (CV) assay, one of the most commonly used *in vitro* biofilm-associated techniques (Table 2), enables optical visualization of biofilm thickness and

TABLE 2 Assays applied for biofilm quantification and viability determination[f]

Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
Fluorescent dyes					
CV	Biofilm matrix biomass		Easy	Dependent on absorption of the dye into the biomass	110, 124, 162
			Inexpensive	Nonspecific to multispecies biofilms	
			Wide applicability	No dimensional information	
				Sample destruction	
				Poor reproducibility	
Congo red	Biofilm matrix biomass		Easy	Low accuracy for biofilm visual analysis	149, 442
			Inexpensive	pH-dependent binding ability	
DMMB	Biofilm matrix biomass	Resazurin, XTT, BTA, FDA	Strain specific (*S. aureus*)	Reagent instability	154, 162
Live/Dead BacLight (Syto 9 and PI)	Semiquantitative	CLSM	Cell viability assessment	Expensive	443, 444
				Intermediate "unknown" population	
				Underestimation of living cells	
				Large no. of samples required	
AO	Apoptotic quantification	Ethidium bromide, epifluorescence microscopy	Time efficient	Lab safety requirements due to high mutagenicity	157, 445
			DNA and RNA labeling		
			Detects apoptotic phenomena		
DAPI	Live-cell biomass	CTC	Feasible combination with other probes	Used only for fixed cells	200, 446
			Nuclear integrity	High concn is required for live-cell staining	
			Cell viability assessment		
XTT	Counts metabolically active cells		Reproducible	Requires highly respirative bacteria	162, 437
			Nondestructive	Variations due to biofilm heterogeneity	
			Cell viability assessment	Time-consuming	
				Large no. of samples required	
AB/resazurin	Counts metabolically active cells		Reproducible	Heat and light sensitive	447, 448
			Cell viability assessment		
CTC	Counts metabolically active cells	DAPI, epifluorescence microscopy	Bright red fluorescence	Detects only highly metabolically active cells	166, 449–451
			Discrimination between active cells and abiotic parts	Toxicity	
			Cell viability assessment	Solute-associated inhibition	
BTA (with phenol red and resazurin)	Counts living cells in biofilm		Inexpensive	Challenging for multispecies biofilm evaluation	167, 452
			No sample manipulation required		
			Cell viability assessment		
FDA	Semiquantitative		Easy	Unsuitable for mature biofilms	162, 437
			Inexpensive	Impaired by biofilm thickness	
			High repeatability		
ATP	Live-cell biomass		Independent of growth conditions	Standard needed to ensure quantification accuracy	453

(Continued on next page)

TABLE 2 (Continued)

Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
			Detects bacteria with low metabolic activity		
			Cell viability assessment		
SYBR Green I	Multispecies biofilm cell quantification	Real-time PCR	Reliable and reproducible	Risk of sample contamination	454, 455
	Can synthesize DNA in real time		No specific probes required		
			Cell viability assessment		
Genetic/molecular approaches					
RT-PCR	Multispecies biofilm cell quantification	Gel electrophoresis (DGGE)	Detects uncultivable or challenging-to-culture species, live and dead cells, matrix components	Risk of sample contamination	456
			DGGE detects predominant species, gives early clinical diagnosis	Expensive and complex procedure	
Real-time PCR	Can synthesize DNA in real time	SYBR green I	Easy, rapid, reliable, and reproducible	Risk of sample contamination	455, 457
	Counts cells in multispecies biofilms		High sensitivity		
			Cell viability assessment		
Next-generation sequencing (NGS)	Quantification of genomic sequences	PCR, RT-PCR[e]	High sensitivity	Expensive	458
			Entire transcriptome available in a single analysis (RNA-seq)[e]		
Proteomic analysis	ECM protein component	Mass spectroscopy/NMR	Biofilm phenotype, protein profile determinant, and resistance pattern analysis	Protein expression variations in multispecies biofilms	187, 190
Microscopy					
FISH	Semiquantitative	CLSM	Independent of growth conditions	Low permeability of DNA probes	175, 200, 459, 460
			Applicable to multispecies biofilms	Low sensitivity	
			Detects all viable microorganisms	Hybridization between complementary PNA probes	
			Visualization and spatial distribution	Expensive and lengthy multistep procedure	
IF	Antibody-antigen complexes	Fluorescently labeled antibodies	Simple procedure	Less flexible procedure	461
				Costly	
				Risk of cross-reaction in multilabeling	
				Complex, with low sensitivity and specificity due to cross-reactions	
				Little reagent standardization and laboratory variations	

(Continued on next page)

TABLE 2 (Continued)

Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
CLSM	Quantitative imaging	Fluorescence assay, FISH, FCS	Nondestructive	Probe efficacy dependent on biofilm EPS complexity	162, 204, 205, 462, 463
			3D imaging Cell and EPS spatial distribution Applicable to thick sample	Special equipment required	
SIM	Live-cell biomass imaging	Fluorescent probes	3D imaging of living cells Enhanced resolution Computational amplification Imaging of thick samples	Specimen instability during multiple-image recording	210, 211, 464
OCT	Biomass, structure, and porosity identification	Ultra-broad-bandwidth lasers	Real-time 3D imaging Speedy measurements Noninvasive Label-free	No cell-level resolution Limited penetration depth	216, 465–467
TEM	Total biofilm matrix biomass imaging		High resolution	Sample prepn required Special equipment required	204, 468, 469
SEM[a]	Synergy with focus ion beam for inner biofilm study	EDS[b]	Surface visualization[a]	Risk of sample distortion due to dehydration[a]	162, 220, 223, 227, 470, 471
ESEM[b]			Detailed 3D visualization[a] No structural damage[b] No sample prepn[b] Imaging of EPS[b]	Low resolution[b] Artifacts due to sample prepn[c]	
Cryo-SEM[c]			No dehydration required[c] Nonconductive surfaces[c] Time efficient[c]	Low resolution[c]	
ASEM[d]			Nanostructure biofilm surface visualization in liquids[d]	Multiple labeling[d]	
STXM	Total biofilm biomass	X-ray fluorescence	Macromolecule distribution	Applicable to thin samples	162, 204, 224, 472, 473
	Chemical biofilm components		Visualization of biological and environmental components and spatial distribution	Special equipment required	
AFM	Chemical biofilm component imaging		Real-time 3D imaging Little/no sample prepn Performed in both air and water Elucidation of molecular interactions High resolution Visualizes cellular interactions	Artifacts and sample damage due to incorrect tip elections Deformation of soft samples Poor image quality in water Special equipment required	227, 228, 474–476

(Continued on next page)

TABLE 2 (Continued)

Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
CRM			High spatial resolution	Low resolution at boundary separation	231, 477
			Species identification	Limited penetration depth	
			Molecular detection		
			Label-free		
MALDI-IMS	Biofilm biomass		Spatial distribution information	Ion imaging artifacts	24, 232, 233
	Chemical biofilm component quantification		Chemical identity definition	Chemical modification of sample surface required	
			Subpopulation discrimination		
			Host-pathogen interaction imaging		
			Differentiation between similar molecules with different masses		
			Imaging over wide mass range		
SIMS	Biofilm molecular composition identification		Molecular identification	Limited mass range	234, 235, 478
			High spatial resolution		
			High depth resolution		

[a]Characteristics referring to SEM.
[b]Characteristics referring to ESEM.
[c]Characteristics referring to cryo-SEM.
[d]Characteristics referring to ASEM.
[e]Characteristics referring to RT-PCR.
[f]CV, crystal violet; DMMB, dimethylmethylene blue; PI, propidium iodine; AO, acridine orange; DAPI, 4′,6-diamidino-2-phenylindole; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; AB, alamarBlue; CTC, oxidized 5-cyano-2,3-ditolyl tetrazolium chloride; BTA, biotimer assay; FDA, fluorescein diacetate; RT-PCR, reverse transcription-PCR; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; PNA, peptide nucleic acids; IF, immunofluorescence; CLSM, confocal laser scanning microscopy; FCS, fluorescence correlation spectroscopy; SIM, structured illumination microscopy; OCT, optical computed tomography; TEM, transmission electron microscopy; SEM, scanning electron microscopy; ESEM, environmental scanning electron microscopy; ASEM, atmospheric scanning electron microscopy; EDS, energy-dispersive X-ray spectroscopy; EPS, extracellular polymeric substances; STXM, scanning transmission X-ray microscopy; AFM, atomic force microscopy; CRM, confocal resonance microscopy; MALDI-IMS, matrix-assisted laser desorption ionization–imaging mass spectrometry; SIMS, secondary ion mass spectrometry.

total biofilm biomass quantification, especially in the initial stages, but is not accurate for calculating cell viability (24). Additionally, CV lacks sensitivity and specificity due to high variability when the dye (i) binds unspecifically to negatively charged molecules or (ii) is unevenly extracted by ethanol. The Congo red agar method investigates coagulase-negative staphylococcus (CoNS) strains for the production of slime and qualitatively scores cellulose and amyloid fiber production by Gram-negative bacteria (tested predominantly in *E. coli*, *P. aeruginosa*, and *Salmonella*) (149–153). Dimethylmethylene blue (DMMB) binds to glycosaminoglycans (GAG) and polysaccharide intercellular adhesins (PIA). This is a species-specific method targeting *S. aureus* biofilms, restraining the diagnostic range of the technique to this PIA-related biofilm matrix-possessing species (154).

The Live/Dead BacLight assay uses double staining with the fluorescent nucleic acid dyes Syto 9 and propidium iodide (PI). Syto 9 fluoresces green and penetrates both damaged and intact cell membranes, providing total cell counts, while PI fluoresces red and crosses only damaged cell membranes. The dual presence of the dyes enables multimodal measurements, including microplate readings, flow cytometry analysis, and even microscopy. Indeed, in flow cytometry studies addressing antimicrobial agent testing for MIC determination, the dual usage of Syto 9 and PI required no preparatory stages. Drawbacks of the green fluorophore, including bleaching, ranging binding affinities (as in the case of Gram-negative bacteria), and background cross-signals, undermine the utility of Syto 9, thus welcoming the combination with PI. Additionally, the pronounced ability of PI to intercalate with DNA results in enhanced fluorescence, displacing Syto 9 and interfering with cell viability testing (155). The large sample requirement makes the method time-consuming and unfit for HT assays (147). Fluorescence-activated cell sorting (FACS) has been investigated intensively for the separation of biofilm subpopulations in the lab, with the potential to be deployed for quantification; reliable single-cell isolation of bacterial cells, on a scale orders of magnitude smaller than what mammalian cell flow systems are commonly designed to provide, remains a confounding factor in many cases (156).

Other nucleic acid-binding dyes applied for fluorescence microscopy include acridine orange (AO), a cell-permeating dye that enables total counts of cells within the biomass; ethidium bromide (EB), which stains nucleic acids red when the integrity of the cell membrane is lost; and the DNA-specific probe DAPI (4′,6-diamidino-2-phenylindole), which stains cells with intact membranes (157–159). The concept of dual-dye flow cytometric cell determination was introduced by use of a customized lab-built device employing a violet diode laser (397 nm) that excites fluorescence of both DAPI and Hoechst dyes in permeabilized and intact cells. Despite the fluorophore complications, flow cytometry is unique for the study of heterogeneous subpopulations (160). A high DAPI concentration is indicative of a thicker biofilm; therefore, it can be used for biofilm extracellular matrix component detection and visualization. Such an example of DAPI implementation is the matrix analysis of *Salmonella enterica* serovar Typhi- and *S*. Typhimurium-associated gallstones (161).

2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) is used to spectrophotometrically identify metabolically active cells that reduce XTT to water-soluble formazan (162). However, it requires bacteria with high levels of aerobic metabolism and exhibits intra- and interspecies variability due to biofilm heterogeneity (30). The resazurin dye, also known as alamarBlue (AB), fluoresces and can be measured via spectrophotometry. In fact, resazurin is a nonfluorescent blue redox indicator that is reduced to the pink fluorescent compound resorufin through cellular respiration (163, 164).

Oxidized 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) dissolves in water, and the electron transport chain of metabolically active cells reduces CTC to fluorescent formazan crystals (165). The incubation time and the CTC concentration affect the number of detectable cells, so this method is suitable only for estimating the number of highly metabolically active cells and only for aerobic or microaerophilic systems (166).

The biotimer assay (BTA) measures metabolism by estimating the time needed for

the visualized color change of specific indicators (phenol red and resazurin) with respect to the initial bacterial concentration according to correlation curves for planktonic bacteria (167). The BTA is a rather simple and convenient colorimetric tool for biofilm susceptibility determination without requiring particular lab equipment. Fluorescein diacetate (FDA) emits yellow fluorescence once it is hydrolyzed by esterases. Although it is used to reproducibly measure microbial activity and biofilm biomass, it is unable to reach deep into thick biofilms (162, 168).

"GRIND AND FIND" APPROACH VIA MOLECULAR ANALYSIS

Multispecies biofilm interrogation requires advanced molecular methodologies that enable species discrimination and strain-specific probes highlighting spatial distribution through advanced microscopy (Table 2). Additionally, surface cell attachment and phenotypic variation are initiated and influenced by specific on/off switch genes. Wild-type strains, isogenic mutants, and plasmids are constructed and studied with molecular assays to further specify and recognize genetic factors involved in biofilm formation. For example, the *ica* gene cluster was found to mediate PIA production, whereas the reversible insertion/excision of the IS*256* sequence element has a strong correlation with phase variation/phenotypic switching of staphylococcal strains (169–173).

PCR-based approaches as well as transcriptional and proteomic profiling can be used to interrogate gene expression differences occurring among free-living and sessile species. Genetic virulence traits are associated with phenotypic shifts contributing to the pathogenesis of clinically relevant strains. Genome sequences for adhesion proteins, tissue-penetrating enzymes, and toxins have been studied extensively for human biofilm-forming colonizers, such as MRSA, enterococcal, *Acinetobacter baumannii*, and *K. pneumoniae* strains (174). Alterations in the expression of transmissibility, QS, and oxidative stress regulatory genes affect the survival of pseudomonal strains in CF patients, and genetic modifications affecting the biofilm-associated pneumococcal phenotype lead to a shift from asymptomatic carriage to nasopharyngeal pathogenesis (175).

Early microarray analysis revealed that during the formation of *E. coli* biofilms and subsequent colonization, 38% of genes were modified, resulting in the altered expression of 600 genes (176). Similar to microarray analyses, quantitative real-time PCR (qPCR) examines a subset of genes from biofilm-isolated cells, while multiplex PCR detects multiple target sequences and is a useful tool for genetic analysis of polymicrobial/multispecies communities or gene polymorphisms (177–179) (Table 3). PCR product detection and quantification are based on probe chemistry.

RNA sequencing (RNA-seq) analyses based on next-generation sequencing (NGS) are now replacing conventional microarrays and provide higher sensitivity and information pertaining to small noncoding RNAs actively participating in oral pathogen dysbiotic processes (180). Additionally, RNA-seq-based transcriptomic analyses have provided a multitude of genes associated with biofilm formation by both Gram-positive and Gram-negative bacterial species (such as MRSA, *Enterococcus faecalis*, and *P. aeruginosa*) in clinical entities, including otitis media, CF, chronic wounds, and endodontic and indwelling device infections (181, 182). NGS platforms provide massive parallel sequencing of DNA segments physically or artificially produced from a single sample. This allows time-efficient reading of entire genomes (whole-genome sequencing [WGS]) as well as identification and quantification of genomic sequences in a sample at the level of a single molecule (target sequencing or sequencing of multiple bacterial species and subpopulations).

NGS in combination with reverse transcription-PCR (RT-PCR) (whole-exome sequencing or RNA-seq) provides evidence for a sample transcriptome. This combinatorial approach does not require genetic sequences, thus worthily replacing microarrays. RNA-seq analyses revealed the proportion of the dormant cell subpopulation within the bacterial biomass of *S. epidermidis* and the coexistence of unknown bacterial species in oral cavity pathogenesis (183–186).

TABLE 3 Biofilm-related gene detection[a]

Method	Species	Gene(s)	Function	Reference(s)
PCR	*Staphylococcus* spp.	*ica* gene cluster	Biosynthesis of PIA, cell accumulation, biofilm formation	444, 479
		MSCRAMM genes	10 genes encoding surface components, involved in recognition of adhesive matrix molecules	480, 481
	Enterococcus spp.	*esp*, *ace*, *agg*, *empfm* operon	*esp* encodes extracellular surface protein (Esp), involved in adhesion and facilitation of colonization and persistence	482, 483
			ace encodes collagen adhesion protein (Ace), which regulates interaction with host matrix proteins (collagens I and IV, laminin)	483
			agg encodes aggregation substance (Agg), involved in conjugation mediation, adhesion to eukaryotic cells, and cellular aggregation	483
			empfm operon is involved in biofilm formation	484
	Escherichia coli	*fimA*, *papC*, *hly*, *ecpA*	*fim* encodes type I fimbriae *pap* encodes P-fimbriae *hly* encodes pilin structure	485
	Salmonella spp.	*adrA*, *csgD*, *csgA*, *gcpA*, *spiA*	*adrA* is involved in cyclic di-GMP level control, cellulose production, biofilm formation *csgD* is involved in biofilm formation *csgA* and *adrA* are involved in curli and cellulose expression *gcpA* is involved in cellulose production, biofilm formation	486
			spiA is involved in biofilm formation and virulence	487
	Acinetobacter baumannii	bla_{PER-1}	Cell adhesion, drug resistance	488, 489
	Pseudomonas aeruginosa	*psl* gene cluster (*pslA* to *-F*)	Involved in biofilm formation, protection against aminoglycoside antibiotics	25, 490
		pel genes	Seven genes involved in pellicle matrix formation	25
		QS genes (*lasIR*, *rhlIR*)	Cell-to-cell communication enhancement, coding for AHL synthase, biofilm formation, resistance, motility	491, 492
		TA genes (*mazEF*, *relBE*, *hipBA*, *ccdAB*, *mqsR*)	Biofilm formation, virulence, "genetic competence"	492
	Klebsiella pneumoniae	*ecpA*	Encodes major pilin subunit	493
		fimA, *fimH*	*fimA* and *mrkA* encode fimbrial subunits	493
		mrkA, *mrkD*	*fimH* and *mrkD* encode minor tip adhesion	493
	Yersinia pestis	*hmsT*, *hmsP* (*)	Regulatory genes, involved in biofilm formation on biotic and abiotic surfaces (*)	494
	Vibrio cholerae	*flaA*, *fliH*, *fliN*, *flgH*, *flgL*, *pomA*	Encode flagellar proteins for cellular motility	
		cpsF, *epsD*, *epsF*, *rfaD*	Encode cell wall components (EPS, LPS)	
		lacI	Encodes a transcriptional regulation protein	
RT-PCR	*Streptococcus pneumoniae*	CSP receptor gene *comD*	Encodes a QS peptide for competence system induction, biofilm formation, virulence enhancement	495
Quantitative RT-PCR	*Streptococcus mutans*	*brpA*, *comDE*, *vicR*	Encode regulatory proteins	496
		gbpB, *spaP*	Adhesion facilitation	
		ftf, *gtfB*, *gtfC*	Polysaccharide synthesis	
		relA	Acid stress tolerance	
		smu0630	Biofilm formation in both presence and absence of sucrose	
	A. baumannii	*pgaABC* gene cluster	Extracellular matrix production and biofilm thickness increase	497
	Y. pestis	*hmsT*	Regulatory gene, biofilm formation on biotic/abiotic surfaces	498
	P. aeruginosa	*psl*, *pel* (*)	Biofilm formation, protection against aminoglycoside antibiotics (*)	25
	Salmonella spp.	*spiA*, *qseB*	*spiA* is involved in biofilm formation and virulence	487, 499

(Continued on next page)

TABLE 3 (Continued)

Method	Species	Gene(s)	Function	Reference(s)
			qseBC are involved in fimbria regulation, biofilm formation, quorum sensing, virulence	
Multiplex PCR	CoNS	*ica* gene cluster	PIA biosynthesis, cell accumulation, biofilm formation	500
Quantitative multiplex PCR		*mecA*, *agrA*, *sarA*, *atlE*, *divIVA* (**)	*mecA* is involved in resistance to methicillin *agr* locus is involved in cell wall and extracellular protein synthesis *sarA* is involved in hemolysin production *atlE* is involved in initial adherence *divIVA* is involved in cell division, is unique to *S. epidermidis* (**)	501 500
	MRSA	*sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, *sej* *eta*, *etb*, *etd*, *cna*, *atl*, *fnbA*, *fnbB* *cap5HK*, *cap8HK*	Staphylococcal enterotoxin genes *eta*, *etb*, and *etd* are exfoliate toxin genes *cna*, *atl*, *fnbA*, and *fnbB* are adhesion genes Surface-associated genes	502
Real-time PCR	*E. faecalis*	*gelE* (***)	Gelatinase production, virulence factor	503
	S. epidermidis	*sarA*, *arlRS*	Encode staphylococcal regulators	504
Quantitative real-time PCR	*S. aureus*	*icaC*, *fnbA*, *fnbB*, *clfB*	Adhesion genes	505
	P. aeruginosa	QS genes (*lasIR*, *rhlIR*) (***)	Production of autoinducer molecules important for cell-to-cell communication, coding for acyl homoserine lactone (AHL) synthase, biofilm formation, resistance, motility (***)	506, 507

[a]PIA, polysaccharide intercellular adhesion; TA, toxin-antitoxin system; AHL, acyl homoserine lactone; QS, quorum sensing; EPS, extracellular polymeric substances; LPS, lipopolysaccharides; CoNS, coagulase-negative staphylococci. Some genes were studied by use of more than one technique, as follows: *, studied with RT-PCR; **, studied with multiplex PCR; and ***, studied with real-time PCR.

The heterogeneous, noncrystalline, and insoluble biofilm extracellular matrix is a rather perplexing assembly. Proteomics has provided deeper knowledge by (i) giving insights into the protein profile determinants that regulate host-pathogen interactions as well as the virulence and pathogenicity traits of the menacing bacteria; (ii) answering substantial questions regarding the biofilm antimicrobial resistance phenotype, offering potential alternatives for rational drug design; and (iii) underpinning physiological differences from planktonic species (187–189). Indeed, the use of proteomics has been substantiated by mass spectrometry (MS) to extend its utility beyond research in the field of diagnostics to clinically oriented infectious disease investigation, with particular emphasis on host-pathogen interactions (190, 191). The coupled use of proteomics with MS was an amenable mapping approach in a landmark study of the microbial proteome and the human microbiome (192). Moving one step further, metaproteomics constitutes a cutting-edge tool for the study of multispecies community interactions by means of cooperation and competitiveness and the ways that these relationships shape the microbial consortium. Metaproteomics also offers the ability to elucidate the metabolic signatures of multispecies biofilms, even for the unculturable bacteria that reside within biofilms but cannot be cultivated by classic microbiological means, potentially providing novel tools for drug design (189, 193, 194).

Hydrogen nuclear magnetic resonance (^{1}H-NMR) has been deployed for proteomic chemical compositional biofilm analysis and exhibits wide applicability to single- and multispecies communities. A "sum-of-the-parts" method to examine *E. coli* amyloid-integrated biofilms aimed to determine the pristine biofilm ECM composition. NMR also allows principal chemical component analysis, supporting the identification of key features between methicillin-susceptible *S. aureus* (MSSA) planktonic and biofilm species. The phenotypic differences were attributed to the uptake of specific amino acids, lipid catabolism, fermentation of butanediol, and metabolism alterations ranging from the production of energy to the accumulation of cellular components

(195). Structural and morphological properties are also characterized by ^{13}C-NMR, which groups carbon pools from a single intact ECM sample. This approach defined the chemical composition and protein content of *Vibrio cholerae* biofilms (196, 197). Proteome-wide tagging and labeling of bacterial proteins allow state-selective analysis and were applied to determine differences in predetermined proteins for planktonic and sessile bacterial species (198). Another example of NMR-based applications for diverse systems involves total correlation spectroscopy (TOCSY)-NMR for the detection of solution and carbohydrate polymer components in *S. epidermidis* biofilms (199). Although NMR is a good choice for biofilm compositional study, in the case of full matrix analysis and determination of the actual composition of biofilm carbohydrate components MS is usually applied due to the need for derivatization into monosaccharides.

IMAGING MODALITIES TO VISUALIZE COMMUNITY ARCHITECTURE

Evolving imaging modalities have contributed to significant improvements in spatial and temporal characterization of biofilms themselves and the signaling factors that alter bacterial behavior within the host-biofilm microenvironment (Table 2).

Optical Microscopy

Fluorescence *in situ* hybridization (FISH) utilizes nucleic acid probes that bind to cRNA or DNA sequences within individual bacterial cells, enabling visualization of the spatial distribution of multispecies biofilms by use of specific probes (16S or 23S rRNA) along with epifluorescence microscopy. The targets are independent of cell metabolism levels, so FISH detects all viable microorganisms, including unculturable ones (200). Peptide nucleic acids (PNA) exhibit higher target specificity and better hybridization kinetics. PNA FISH is versatile in clinical microbiology and biofilm investigations, in combination with confocal laser scanning microscopy (CLSM), offering species identification, spatial information, and bacterial load determination within mixed-species biofilms in chronic wound specimens and CF sputum (201, 202). On the other hand, immunofluorescence (IF) assay employs fluorescently labeled antibodies that bind to specific target antigens and detect cell types or cellular subcomponents defining expression at biofilm developmental stages (203).

The use of specific fluorescent probes has enhanced the specificity of CLSM for protein quantification and localization within colony biofilms. The structural complexity of the microbial polysaccharides confers a significant limitation on the use of labeled probes (45, 204, 205). Fluorescent probes (dextrans, rhodamines, and Oregon Green) and fluorescence correlation spectroscopy (FCS) combined with CLSM enable biofilm diffusion quantification and thickness evaluation. These provide information on factors affecting antibiotic and biocide transportation within the biofilm microenvironment (206). CLSM in combination with computational software can be extended to a multispectral and three-dimensional (3D) biofilm imaging and quantification technique. Digital analysis has replaced conventional qualitative and semiquantitative techniques with more accurate and sensitive procedures. Computer programs and software packages for biofilm visualization, quantification, and deconvolution (Imaris, COMSTAT, Amira, Volocity, ISA3D, ImageJ, and Fiji), containing structural variables including porosity, thickness and roughness coefficients, fractal dimension, and homogeneity, have been launched or belong in the public domain, thus providing analysis options (207–209).

Structured illumination microscopy (SIM) enhances fluorescence abilities for 3D imaging of living cells (210). Computational removal of out-of-focus light leads to true optical sectioning via exclusion of the associated blurred images (211–213). 3D-SIM visualizes macromolecules interacting within the surrounding cellular environment.

Optical coherence tomography (OCT) offers real-time, 3D *in vivo* biofilm imaging ideal for biomass structure development and porosity identification. OCT enables nondestructive *in situ* biofilm analysis for chronic middle ear infection and dental

biofilm growth monitoring (214, 215). Doppler OCT imaging can be employed for quantitative biofilm dynamics analysis during the biofilm formation stages (216).

Electron and X-Ray Microscopy

Transmission electron microscopy (TEM) offers a high resolution for visualizing specific structural components, such as polysaccharide fibrils and extracellular DNA. Combined with ruthenium red staining, TEM reveals the morphological features of bacterial cells within a fibrous matrix; such an example is the confirmation of the presence of *P. aeruginosa* biofilm in a burn wound animal model (204). Scanning electron microscopy (SEM) is applied for biofilm structure and function (morphology, glycocalyx density, and layer thickness) analysis on living surfaces or inanimate materials. Compared to TEM, it is considered the optimal imaging tool, offering detailed, high-resolution 3D biofilm visualization (217). *Staphylococcus lugdunensis* and *Propionibacterium acnes* causing implant-related osteomyelitis in an *in vivo* animal model were visualized by SEM analysis when present in intercellular aggregates within the bone marrow but not when localized intracellularly (218).

Unlike SEM and TEM, environmental SEM (ESEM) retains visualization ability without laborious sample preparation and facilitates imaging of external cell polymers, though the spatial resolution is reduced compared to that of traditional SEM techniques (217, 219). ESEM combined with energy-dispersive X-ray spectroscopy (EDS) has been employed for determination of the *Proteus mirabilis* biofilm composition on infected urinary catheters (219). Finally, cryo-SEM is optimal for fragile, fully hydrated samples, offering ultrafast freezing as an optimal method of nonsolid specimen fixation (220). When biofilms from diabetic foot wounds were investigated using SEM, a high level of resolution and detail was obtained, but the exopolymer matrix was destroyed during sample preparation. This limitation was overcome by the application of ESEM or cryo-SEM due to preservation of the biofilm hydrated state (221).

The recent discovery that ionic liquids provide clear SEM-based visualization of biofilms from fully hydrated biological samples has opened new ways for broad SEM utilization (222). Additionally, the method of immersed atmospheric SEM (ASEM), combining heavy metal labeling, charged nanogold labeling, and immunolabeling, enabled visualization of nanostructures of the biofilm-surface interface in liquids (223).

Scanning transmission X-ray microscopy (STXM) provides data on spatial distribution as well as quantitative and qualitative analyses of biofilm components, thus dissecting the heterogeneity of microbial communities (224). For example, multispecies microbial consortia have been investigated by STXM for the analysis of selenium particle biotransformation (225). In another application, a CLSM-STXM combination was used on pseudomonal biofilms tested with antimicrobial agents, such as chlorhexidine dihydrochloride, benzalkonium chloride, triclosan, and trisodium phosphate, to detect differences in cell density, spatial distribution, and membrane integrity (226).

Scanning Probe Microscopy and Imaging Mass Spectrometry

Atomic force microscopy (AFM) has gained increased attention due to its microbial cell surface structural and physical probing ability (227, 228). AFM applications involve visualization of the biofilm structure and detection of the physicochemical interactions (van der Waals and electrostatic forces) between microbial cells and various surfaces (229). For example, an AFM-mediated investigation of *P. aeruginosa* biofilm formation and adhesion on surface substrates, including sheets of aluminum, steel, rubber, and polypropylene, indicated that the polypropylene substrate's rough surface exhibited enhanced bacterial adherence compared to that on steel (230). Confocal resonance microscopy (CRM) determines chemical differences among bacterial species. This technique can be used to map distributions of biomass, EPS, chemical components, and molecular compounds (231).

Matrix-assisted laser desorption ionization–imaging mass spectrometry (MALDI-IMS) provides the spatial molecular distribution for each identified molecule within the biomass (24). IMS generates a wealth of data for developing "chemical fingerprint"

databases that enable chemical definition of colonies between bacterial species as well as discrimination of the subpopulations within biofilms (50, 232, 233). MALDI-IMS was used on single-species UPEC biofilms in order to analyze differences in the spatial proteome of adhesive fibers that confer virulence and biomass growth. Phase variation of the promoter of *fim*, encoding type 1 pili, was found to be under the control of oxygen availability (24).

Secondary ion mass spectrometry (SIMS) constitutes a label-free imaging methodology providing the highest depth and spatial resolution (234, 235). Unlike MALDI-IMS, SIMS does not require the use of a matrix to get spatial information. When complemented with MALDI and CRM, this method provides identification and specific locations of proteins and other chemical species, macroscopic and cell-level chemical differences, mass-based discrimination of similar molecules, and the spatiotemporal distribution of metabolites and signaling molecules, even for multispecies biofilms (234–236). For example, the chemical heterogeneity and secondary metabolites, such as rhamnolipids or quinolones, participating in biofilm growth and cellular signaling of a *P. aeruginosa* wild-type strain and an isogenic QS mutant were investigated by MALDI-SIMS, providing *in situ* chemical mapping (234).

The future of biofilm visualization requires (i) *in situ* imaging of fully hydrated biological specimens, with avoidance of biofilm-destructive procedures; (ii) defined probes that enable a combination of imaging techniques for better resolution and 3D structure and/or microscopy that does not require probes (OCT or magnetic resonance imaging [MRI]); and (iii) updated software tools to meet the demands for enhanced digital biofilm imaging analyses (209).

MODELING BIOFILMS *EX VIVO*

Ex vivo models involve biofilm growth on natural tissue in a minimally altered environment, offering more strictly controlled experimental conditions for extensive research than those of *in vivo* models (5). They facilitate the study of the association between biofilm formation and virulence or determinants of pathogenicity. These models contribute to the development of effective imaging tools that can monitor tissue-specific bacterial establishment. For example, porcine skin explants infected with *S. aureus* and *P. aeruginosa* have been used for the growth of mature biofilms that mimic chronic skin wounds and provide a way to assess antibiofilm antibiotic efficacy (237).

Implants and medical devices enable biofilm formation that results in chronic infection. An *ex vivo* whole-blood model of *S. epidermidis* prosthetic joint infection was employed to elucidate the association between complement C5a levels and PIA-related biofilms as one of the main components of biofilm accumulation (238). An *ex vivo* model of MG63 human osteoblasts cocultured with *S. epidermidis* strains was used to examine whether infection-related isolates exhibit adherence and internalization abilities different from those of commensal isolates. Flow cytometry, BRT, and CV assays were combined to test this hypothesis, which showed no statistically significant difference compared to studies on the harboring and expression of virulence factors that efficiently discriminate invasive strains from commensals (110, 239).

Ex vivo models are also useful for assessing therapeutic windows of antibiofilm experimental treatments. A 3-day tolerant *S. aureus* biofilm grown on a porcine skin explant model was no longer detectable after treatment with a surfactant-based wound dressing, whereas biofilms wiped with moistened gauze reoccurred (240). In a similar model, biofilms formed within the first 24 h were found to be more susceptible to antibiotics than mature formations, validating current therapeutic strategies that aim at early wound prophylaxis (241). Similarly, porcine skin models have been used to test the effect of negative-pressure wound therapy with instillation on *P. aeruginosa* biofilms as well as the influence of tetracycline release of a zein and poly-ε-caprolactone (zein/PCL) fibrous dressing on *S. aureus* biofilms (242, 243). The comparative efficacy of glycine and tricalcium phosphate (TCP) over that of glycine or sodium bicarbonate in biofilm removal was examined by use of an *ex vivo* model using biofilms grown on

titanium (Ti) and zirconium (Zr) implant surfaces (244). The effects of topical treatments and commonly used antimicrobial dressings on biofilms of different maturation levels were also tested (237, 245).

Cardiac valves constitute a substratum for *in vivo* clinical biofilms, highlighting the need for an *ex vivo* study model. An *ex vivo* model of porcine cardiac valve tissue combined with electron microscopy was used to examine the effects of the aggregation substance (Asc10) protein of *E. faecalis* on biofilm formation and persistence in endocarditis. It was found that Asc10 increased cell aggregation, leading to accelerated biofilm formation (246).

The murine respiratory pathogen *Mycoplasma pulmonis* was recently used for biofilm development of a tracheal epithelium organ-mounting system that can be scanned with a fluorescence microscope, resulting in the observation that *in vitro* and *ex vivo* biofilms share common structural features and characteristics (247). In a similar way, an *ex vivo* pig model of bronchiolar tissue infected with *P. aeruginosa*, resembling the CF lung mucus environment, provides a realistic and replicable HT assay to study the structure as well as virulence and physicochemical traits present in chronic biofilm-associated lung infections (248).

The otitis, nasal, and throat mucosal biofilm formation models are further substrates for *ex vivo* biofilm model applications (249). An *ex vivo* model of stainless steel tympanostomy tubes inoculated with *P. aeruginosa* and *S. pneumoniae* combined with SEM was used to examine biofilms formed on clearance of mucoid plugs after ofloxacin challenge (250). Moreover, consecutive lavage samples of otitis media biofilms revealed the presence of NTHi subpopulations with different growth rates and gene expression modes (251). In the case of the oral cavity, an *ex vivo* root canal model was applied to test the parameters influencing the efficacy of irrigation in biofilm removal, utilizing marketed fibronectin- and collagen-based films (252, 253).

In a GI biofilm study, the pathogenicity of *Aeromonas caviae* strains isolated from human feces was investigated by use of an *ex vivo* rabbit ileal and colonic mucosa model. Cultured tissue was used for adhesion assessment of *A. caviae* strains (incubated with colonic and ileal intestinal fragments), whereas microscopy generated information regarding colonic and ileal mucosa colonization and biofilm formation (254).

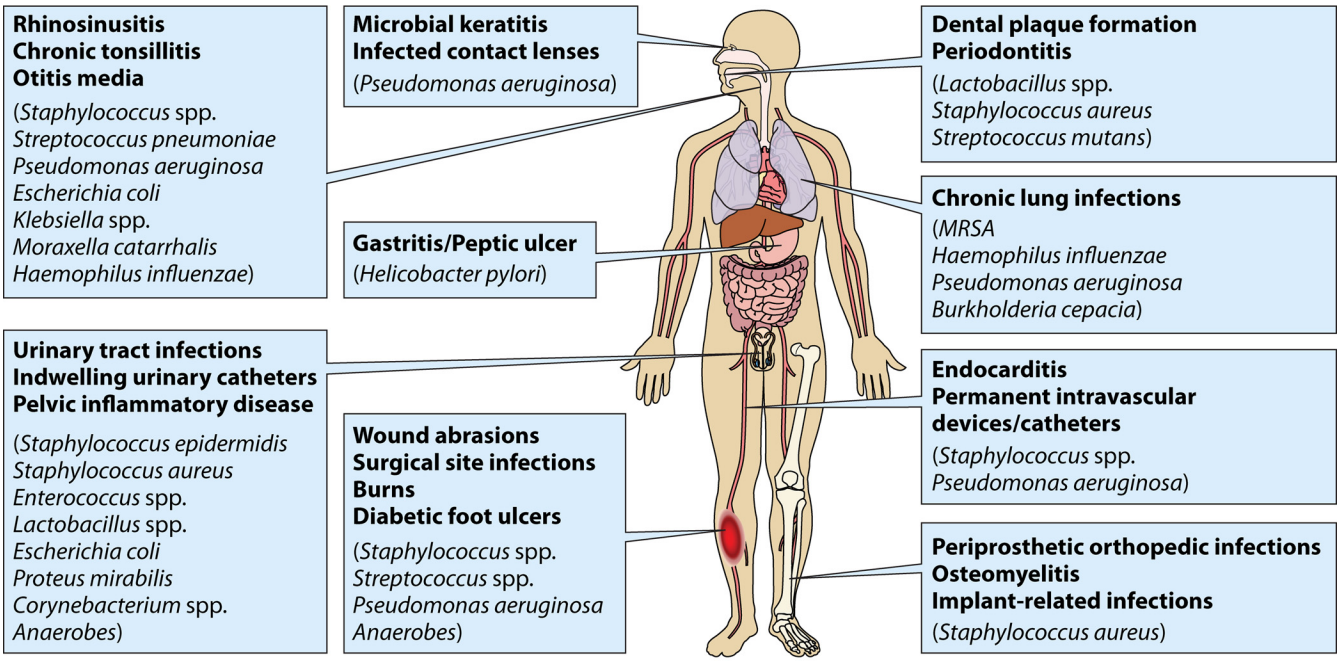
A porcine vaginal mucosal model (PVM) aimed to investigate the interactions among the commensal vaginal *Lactobacillus* spp., the anaerobic species *Gardnerella vaginalis*, and the sexually transmitted organism *Neisseria gonorrhoeae*, as well as the mechanisms of biofilm formation. The model quantified and unraveled different profiles for the effects of pH, acids, and *Lactobacillus crispatus* on *G. vaginalis* and *N. gonorrhoeae* growth when a live vaginal mucosal surface was used (255).

DISSECTING BIOFILMS *IN VIVO*

The urgent need to investigate biofilm-associated infections and develop effective therapeutic strategies gave birth to translational approaches that allow the dissection of virulence and pathogenicity determinants and the identification of novel therapeutic targets. The widespread host-based models simulating biofilm-related mammalian diseases (Fig. 4) are discussed below.

Nonvertebrate Animal Models

Exploiting nonvertebrate animal models has provided an important solution to the need for investigations of biofilm-associated infections. The fruit fly (*Drosophila melanogaster*), the wax moth (*Galleria mellonella*), and the nematode worm (*Caenorhabditis elegans*) have historically been used to evaluate microbial virulence traits involved in mammalian infections and to test the efficacy of antimicrobial compounds (256–262). These studies have elucidated factors affecting virulence, pathogenicity, and host immune responses by altering gene expression, studying efflux systems, toxins, or QS compounds, and allowing easy measurement of the host mortality (263–270). The majority of pathogenic assays using these invertebrate models, by design, detect



	Model organisms	Pathogen tested	Associated infections
Non-vertebrate animal models	Fruit fly	*Pseudomonas* spp. *Providencia sneebia* *Yersinia pestis*	Epithelial/Wound/Gut infection
	Wax moth	*Pseudomonas aeruginosa* *Acinetobacter baumannii* *Burkholderia cepacia* *Burkholderia multivorans* *Campylobacter* spp.	Sepsis Visceral invasion
	Nematode	*Pseudomonas aeruginosa* *Escherichia coli* *Burkholderia pseudomallei* *Burkholderia cepacia (Bcc)* *Yersinia pseudotuberculosis*	Pulmonary infection
Mammalian Animal models	Zebrafish	*Streptococcus suis* *Mycobacterium haemophilum* *Pseudomonas aeruginosa* *Vibrio parahaemolyticus*	Meningitis Septicemia Visceral organ granulomatosis Chronic inflammation Gut infection
	Rat/ Mouse	*Staphylococcus aureus* *Staphylococcus epidermidis* *Enterococcus* spp. *Mycobacterium bovis* *Pseudomonas aeruginosa* *Escherichia coli* *Vibrio vulnificus* *Acinetobacter baumannii* *Porphyromonas gingivalis*	Burn, abscess, wound, arthritis, respiratory tract/gut/ implant/surgical site infection, catheter-associated urinary tract infection, periodontitis, osteomyelitis

FIG 4 Comparative visualization of biofilm-attributed human infections and classes of major vertebrate and nonvertebrate models developed. This is not an extensive list, but presentation of the bacterial strains that are most commonly encountered in biofilm-related research studies is included.

survival rates despite the wealth of microscopy and molecular methodologies that are often employed as surrogates for intensive interrogation.

Drosophila is extensively used to model wound or epithelial infections by Gram-negative bacteria, and comprehensive methodology exists for modeling persistent colonization and for assays of persister cell formation (271–273). For the adult fly, the infection methodology includes (i) needle pricking (mimicking wound infection), (ii) feeding (mimicking oral infection methods), and (iii) injection pumping (mimicking a systemic infection similar to bloodstream infection in mammals). Larvae are less commonly infected than adults with either the feeding or injection pumping method (271–273).

Innate host responses in *Drosophila* against *P. aeruginosa* are largely conserved in humans (274–276). It was also demonstrated that there is a remarkable conservation in the virulence factors used by bacteria to infect both *Drosophila* and mammals (277, 278). *Drosophila* models remain valuable tools for exploring biofilm molecular determinants; two adherence factors required for *in vivo* virulence of *Pseudomonas fluorescens* are (i) *gmd*, encoding the enzyme GDP-mannose dehydratase, involved in the synthesis of A-band-O-antigen-containing lipopolysaccharide (LPS); and (ii) a *fadL* homologue involved in long-chain-fatty-acid transport (279). *D. melanogaster* has served as a model for the study of the functional correlation of the paraoxonase family members with biofilm formation and QS *in vivo*. This correlation has shown the protective role of the human enzyme paraoxonase 1 (PON1) in innate immunity (280). Finally, *Drosophila* as an infection model of *P. aeruginosa* biofilms has provided a direct or indirect model of virulence and pathogenesis determinants through comprehensive analysis of the molecular responses of hyper- and low-biofilm-forming strains (281).

The fly has also proven valuable for modeling of biofilm formation by *Providencia* spp.; it was identified that *Providencia sneebia* is lethal while propagating in the fly but elicits a mild immune response (282). Moreover, a chronic gut *Yersinia pestis* infection was established in the anterior fly larva midgut to mimic and dissect the relationships between biofilm-associated genes (PhoP, GmhA, and OxyR), the gut immune system, and antimicrobial peptides (283).

Galleria mellonella, a worm with a complex immune system, provides a competitively advantageous alternative to other hosts regarding size and amenability for the evaluation of antimicrobial treatments (284). The infection development model employs larval caterpillars (third- or final-instar stage) that are injected with bacteria in the hemocoel via the last left proleg (285). The wax moth has (i) facilitated screening of a U.S. Food and Drug Administration-approved library to identify antibiofilm compounds against *Francisella novicida* (286); (ii) been used to model biofilm formation by *Acinetobacter baumannii*, *Burkholderia cepacia*, *Burkholderia multivorans*, *Campylobacter* spp., and species in polymicrobial infections, such as the *P. aeruginosa* Liverpool epidemic strain and oral streptococci (287–292); and (iii) been applied for evaluation of the antibacterial and antibiofilm activities of alternative therapeutic strategies against a multitude of pathogens, including *S. aureus*, MSSA, MRSA, and *Acinetobacter baumannii* (293–295).

The free-living nematode *C. elegans* has been used to model infections by most Gram-negative bacteria, including *E. coli*, *Burkholderia pseudomallei*, *B. cepacia* complex (BCC), *P. aeruginosa*, and *Yersinia pseudotuberculosis*, through the feeding methodology (296–300). The *C. elegans*-BCC interaction studies provided insights into the identification of the roles of specific biofilm-related virulence factors, including (i) the autoinducer-dependent acyl-homoserine lactone (*aidA*), (ii) the phenazine biosynthesis regulator (*pbr*), and (iii) the host factor phage Q (*hfq*) surface-associated lipoproteins (261, 301–307).

A few reports involve less common invertebrate hosts for modeling of biofilm infections, such as (i) the ciliated protozoan *Tetrahymena pyriformis* for *Legionella* sp. and *K. pneumoniae* biofilms, (ii) the amoebae *Acanthamoeba* spp. for *Legionella* sp. and nontuberculous mycobacterium (NTM) biofilms, and (iii) the soil-living amoeba *Dictyostelium discoideum* for *A. baumannii*, *Legionella* sp., *P. aeruginosa*, and *S. aureus* biofilms

(308–314). There have been attempts to employ plants, such as the duckweed *Lemna minor* and the wounded alfalfa *Medicago sativa*, to allow biofilm growth of *Burkholderia cenocepacia* and *S. enterica* (315–317). *Arabidopsis thaliana* (thale cress) is a popular tool in plant molecular biology that has been utilized as a host system to study *B. subtilis* and *P. aeruginosa* biofilm formation (318, 319).

Vertebrate Animal Models

Vertebrate animal models have been used extensively to mimic human biofilm infections and to test antimicrobial efficacy (320, 321). Localized mammalian animal models may refer to skin and soft tissue infections experimentally studied by use of infected excisional wounds, partial-thickness abrasions, scratches, burns, abscesses, and surgical sites. Apart from the pathogen of interest, experimental variation can also include the mammalian host by alteration of its immunological state (5, 266, 321–338). Here we include some examples from the literature which underline the recent efforts and achievements in the field of vertebrate-based biofilm formation and evaluation.

The optically clear zebrafish, *Danio rerio*, has been applied for *P. aeruginosa* biofilm imaging and for biofilm formation quantification of the bacterial fish pathogen *Edwardsiella tarda* and the pig pathogen *Streptococcus suis* (339–342). Zebrafish has also been used to study *Mycobacterium haemophilum* and to evaluate oral pathogen adhesion in a vertebrate orointestinal model (343, 344).

Mouse model biofilm formation has been described for (i) a multidrug-persistent *A. baumannii* murine wound infection to evaluate therapeutic solutions against trauma and surgical infections in hospitalized patients (345) and (ii) BALB/c and C3H/HeN mice to study chronic *P. aeruginosa* wound infection establishment after a third-degree burn with skin necrosis (346).

Reports for *in vivo* porcine skin infection models are surprisingly scarce given, for example, the MRSA-associated skin wound model that examines the effective bacterial load reductions of various methods of debridement (hydrosurgery and plasma-mediated bipolar radiofrequency ablation) (347). Antimicrobial photodynamic therapy (aPDT) has been applied against a variety of pathogens implicated in acute and chronic biofilm-associated infections. In order to test the efficacy of photosensitization, various animal models have been applied. Mouse, rat, and pig wound, osteomyelitis, and arthritis models as well as dog dental infection models revealed the therapeutic potential of PDT against pathogens, including streptococci, MSSA, MRSA, *P. aeruginosa*, *A. baumannii*, *P. gingivalis*, and *Fusobacterium nucleatum* (322–327).

Rat models are in constant use due to their amenability to wound and infection site colonization without the requirement of disturbing factors (foreign bodies or diabetes mellitus induction), allowing a correlation with rat age, location of the wound, and size of the inoculum (348). Mouse models employing tissue cages and catheter infection can provide information about the antimicrobial efficacy of biomaterial coatings and host-pathogen interactions (349, 350). In the field of implanted device-associated infections, a major model involves totally implantable venous access ports (TIVAPs) implanted in rats. This model allows monitoring of bacterial biofilm development, physiology, and prevention strategies by use of inocula of four bioluminescent pathogens, including *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* (351). Along with rat models, rabbits have also been used in prospective animal studies to investigate, for example, the effect of a 60-μA implantable direct-current fusion stimulator on the implant-related infection rates in a postoperative spinal wound infection model. All sites were inoculated with MSSA, but no significant difference was observed for the implant and bone infection rates and the bacterial load (352). Another observation found by use of an indwelling device rat model was the inhibitory effect of QS disruption via RNAIII-inhibiting peptide (RIP) on *S. epidermidis* infections (333).

Titanium implants have been used to examine the inhibitory and prophylactic effects of gentamicin coatings on titanium oxide surfaces (bioactive TiOB) (353). Likewise, murine osteomyelitis models have been developed to monitor immune responses occurring during *S. aureus* infection and healing (interleukin-4 [IL-4] and

gamma interferon) following implant placement after bone fractures. A fracture fixation murine model employed skeletally mature C57BL/6 mice that were treated with Ti fracture fixation plates and screws after femur osteotomy (354). A rat model of acute foreign-body osteomyelitis was used to evaluate the role of the *E. faecalis ahrC* and *eep* genes in biofilm formation and virulence. Stainless steel orthopedic wires were inoculated with *E. faecalis* OG1RF Ω*ahrC* and Δ*eep* isogenic mutants and implanted into the proximal tibiae of rats (355). In an *S. aureus*-associated murine osteomyelitis model, a fixation plate was employed for debridement of the fracture during a revision surgery, followed by the placement of a vancomycin-laden implant. Infection monitoring was achieved via bioluminescence imaging, X-ray, and micro-computed tomography (μCT) for the evaluation of both osteolysis and bone formation (328).

An implantable-cage MRSA infection model using male albino guinea pigs was developed, and treatment efficacy against planktonic and biofilm infections was evaluated for the most commonly prescribed antimicrobial agents. Antimicrobial testing concluded with an optimal combination of fosfomycin and rifampin against implant-associated MRSA infections (356). Rabbits have also been used in models of spinal implant *S. aureus* infections to assess the antimicrobial potential of modified Ti pedicle screw coatings as well as the efficacy of front-line antistaphylococcal drugs (357, 358).

The majority of models developed for the study of gastrointestinal colonization and biofilm formation are murine, with an emphasis on colitis. One of these mouse models identified the importance of the stringent response regulator DksA for *Salmonella* pathogenicity, virulence, and biofilm formation. During early stages of *S*. Typhimurium infection, DksA is induced at the murine midcecum and is required for systemic infection (359). Likewise, the contribution of other bacterial species to early GI colonization was proved by use of a *Clostridium difficile* colon and cecum mucus mouse model with FISH application (360). A notable reported intestinal model involves evaluation of *Shigella flexneri* adhesiveness in the guinea pig gut. Deficient LPS inner core biosynthesis presented by the Δ*rfaC* mutant resulted in enhanced biofilm formation on and adhesion and invasiveness to human epithelial cells, an observation that could be utilized for the development of new therapeutics. It was also concluded that fitness gains through host adhesion and strong biofilm formation did not replace the effect of fitness loss due to LPS deletion on survival rates (361).

Mouse models have also been applied to mimic biofilm-associated UTIs as well as to evaluate the correlation between host immune systems and local defense factors, the attributed infection (nephron obstruction and pyelonephritis), and treatment (362). Future UTI models may utilize human-mouse chimeras based on severe combined immunodeficient (SCID)-hu mice in order to achieve a better match with human conditions (321).

P. aeruginosa is of utmost importance in chronic lung infection, and efforts to reveal the relationships between the vertebrate host and virulence factors are long-standing (329). A murine inhalation model was developed to connect chronic *P. aeruginosa* nasopharyngeal carriage with lung infection, which complicates and increases resistance to therapeutic agents (363). In a *P. aeruginosa* and *S. aureus* coinfection mouse CF model, *P. aeruginosa* isolates were found to outcompete *S. aureus* at early stages of chronic infection (364). Moreover, the therapeutic potential of inhaled liposomal amikacin was tested in a *P. aeruginosa* rat lung infection model (330). *P. aeruginosa* biofilms formed on murine tumors have also been used to examine the efficacy of ciprofloxacin, colistin, tobramycin, and their combinations (331).

Oral biofilms are a major chapter in biofilm-related literature, and the quest for animal models in closer proximity to humans leads to the exploitation of primates. In a screening effort to identify an optimal host for monitoring *Aggregatibacter actinomycetemcomitans*, the leading cause of periodontitis in humans, *Macaca mulatta* (rhesus [Rh] monkeys) ranked as the first choice. Rh monkeys provide an established oral habitat to validate *A. actinomycetemcomitans*-mediated periodontitis (365). Rh monkeys have also been tested for age-mediated apoptosis gene expression in oral mucosal tissues. The transcriptomic analysis of apoptotic gene expression reflects

decreased apoptotic phenomena in the oral mucosa of aging animals that consequently may increase dysregulation in anti-inflammatory responses and induce disease (366).

A murine periodontitis model combined with genomics and quantitative PCR was used to examine the role of host factors in persistent subgingival biofilms (336). Likewise, a rat model of *A. actinomycetemcomitans*-induced periodontitis was combined with RT-PCR for versatile applications, ranging from genetic reduction to therapeutic efficacy evaluation according to exopolysaccharide variations (367). Another rat model, the *in vivo* extraradicular biofilm model, is more inclusive, as it may facilitate identification and quantification of biofilm-forming bacteria by use of real-time PCR (rt-PCR) and micro-computed tomography (368). Chinchillas (*Chinchilla lanigera*) have been employed for study of middle ear infections in an attempt to establish realistic infection models, to overcome limitations in biofilm analysis, and to correlate the sequence of events in polymicrobial infections with the host immune system. A chinchilla otitis media model involving an NTHi 86-028NP isolate and an isogenic phosphorylcholine (PCho) transferase (*licD*) mutant was deployed to show that PCho facilitates biofilm stability and alleviates the host immune response, promoting NTHi infection and increased persistence (369).

Biofilm treatment efficacy testing is a field that demands available surfaces and assays, and rabbits and guinea pigs have been tested toward this end. A rabbit otitis model examined the efficacy of a nanoporous middle ear implant coating releasing ciprofloxacin against *P. aeruginosa* (370). In another study, a guinea pig animal model combined with otoendoscopy, histology, and bone CT was used to examine the inhibitory effect of a vancomycin-eluting nanofiber mat against MRSA biofilms formed on ossicular prostheses and middle ear infections (371).

Animal models offer insight into the interplay of basic biofilm features and host defense mechanisms (372). However, the risk of coincidental assessment of the experimental conditions is high, and most mammalian models are considered not identical in effectively reproducing the major infectious entities within the human host (Fig. 4).

IN VIVO IMAGING TOOLS

Bioluminescence imaging takes advantage of bacterial cloning vectors optimized to allow for the expression of luciferin in different bacterial cells (373). As a result, these microbial cells "glow in the dark," and a sensitive imaging camera is able to capture images of small animals, revealing both the location and intensity of the infecting microorganisms in real time, in a noninvasive manner. This technology has dramatically reduced the number of animals needed to obtain statistically significant data on antimicrobial therapeutics (374). Previously, animals were generally sacrificed at discrete time points, followed by tissue removal, homogenization, and CFU culture and enumeration. This has often left unanswered the question of what happens at later times, as many antimicrobial treatments are highly effective at early time points but the microbial cells regrow when the initial antimicrobial action has ceased (351, 375). Scarce protocols examine the dynamic processes of biofilm formation, bacterial load, infection physiology, and response to treatment for *in situ* models of implantable devices. Bioluminescence combined with a totally implantable venous access port model has been used to assess localized and systemic infections related to CVC in rats, reproducing clinically significant situations of foreign body-associated infections (351). These models have been valuable in the investigation of aPDT, which involves photosensitizing dyes topically applied to the infection site and subsequent harmless visible light illumination and reactive oxygen species (ROS) generation (324, 376). Bioluminescence imaging of localized infections not only is well suited for monitoring the effectiveness of experimental antimicrobial therapeutics but also has a major role in the study of microbial virulence and pathogenicity. A virulence-specific study of an oral mouse infection model that employed a multiplexed biophotonic imaging-based inducible luciferase reporter was used to track individual species temporally and spatially in polymicrobial biofilms (377). Bioluminescence technology has expanded to

include imaging in three dimensions for biofilm infections that would otherwise be challenging to monitor and treat (378–382).

Near-infrared fluorescence molecular probes have been delivered by local injection and used to visualize inflammation as well as implant-related biofilm infections in a rapid and minimally invasive manner, therefore aiming at detecting orthopedic-related infections (383). The hydro-sulfo-Cy5 probe detects ROS generation following bacterium-free or biofilm-containing implant application. On the other hand, diaminocyanine sulfonate was used to detect *ad hoc* biofilm-associated nitric oxide production (384).

The dye C-SNARF-4 coupled with ratiometric imaging allowed 3D visualization in real time of the extracellular pH variation in the growth of dental biofilms (385). Ligand-targeted ultrasound contrast agents (UCAs) coupled with optical and high-frequency acoustic microscopy have facilitated detection, visualization, and quantification of *S. aureus* biofilm matrices in both test tube and animal model adjusted surface cultures (386). Noninvasive 3D OCT can be used to identify as well as visualize, in real time, *in vivo* bacterial microcommunities involved in biofilm-associated otitis media (387). OCT has been coupled with low-coherence interferometry to image the biofilm layer on the sensitive, ultrathin tympanic membrane in the middle ear (388). Another optical technique, intraoral cross-polarization swept-source OCT (CP-OCT), provided an additional tool to visualize the *in vivo* density of the biofilm formed between the enamel and the interface (389). Spatially resolved MRI was used to study structures within dynamic physical and chemical material systems, and also in biological systems, with a limited number of biofilm applications (390). In an analogous fashion, micropositron electron tomography coupled with the radioactive probe [^{18}F]fluorodeoxyglucose was used to monitor *S. aureus* biofilm infections and antimicrobial therapy in a mouse model (391).

Optical, radiographic, and μCT imaging modalities have been used as tools to monitor orthopedic implant biofilm infections longitudinally, to assess the bacterial load, and to detect osteolysis (392). Multiresolution imaging coupled with *in vivo* labeling uncovered the supplemental tasks of the matrix components of *V. cholerae* biofilms (393). Maltodextrin-based imaging probes (MDPs), a family of light-emitting contrast agents chaperoned with maltohexaose, enable *in vivo* bacterial detection with paramount responsiveness through a cell-specific mechanism. MDPs are rapidly internalized through the maltodextrin transport pathway and selectively accumulate within bacteria at low concentrations, with enhanced specificity over that for mammalian cells (394).

Finally, a bioluminescence-fluorescence combination containing a naturally light-emitting bioluminescent *S. aureus* strain and fluorescent neutrophils from an enhanced green fluorescent protein (EGFP)-expressing mouse strain (LysEGFP) was applied for visualization of a surgical site infection in the knee joint in a mouse model. *In vivo* bioluminescence imaging was used to quantify the microbial burden. Accordingly, *in vivo* fluorescence imaging was used to assess the neutrophil inflammatory response. In the same mice, bioluminescence and fluorescence optical imaging combined with μCT imaging allowed for visualization of 3D anatomical details. This triple imaging combination was proposed to simultaneously track bone biofilm infections, including inflammatory responses and local anatomical modifications, in a noninvasive manner (381).

SHAPING UP THE METHODOLOGICAL PIPELINE

Literature Search on Method Implementation

Extensive electronic literature searches provided additional information on the current methodological landscape of biofilm research. Queries were conducted to search the literature from the first biofilm report in October 1988 through August 2017 by using the keywords "biofilm(s) and/or assay" (particularly involving the colorimetric assays applied in the lab) and "biofilm(s) and/or device" separately (Fig. 5). Two specialized exceptions, for "microfluidics" ("bioflux" and "microfluidic device") and "Calgary" ("MBEC device" and "MBEC HT"), were introduced to the query search. Articles

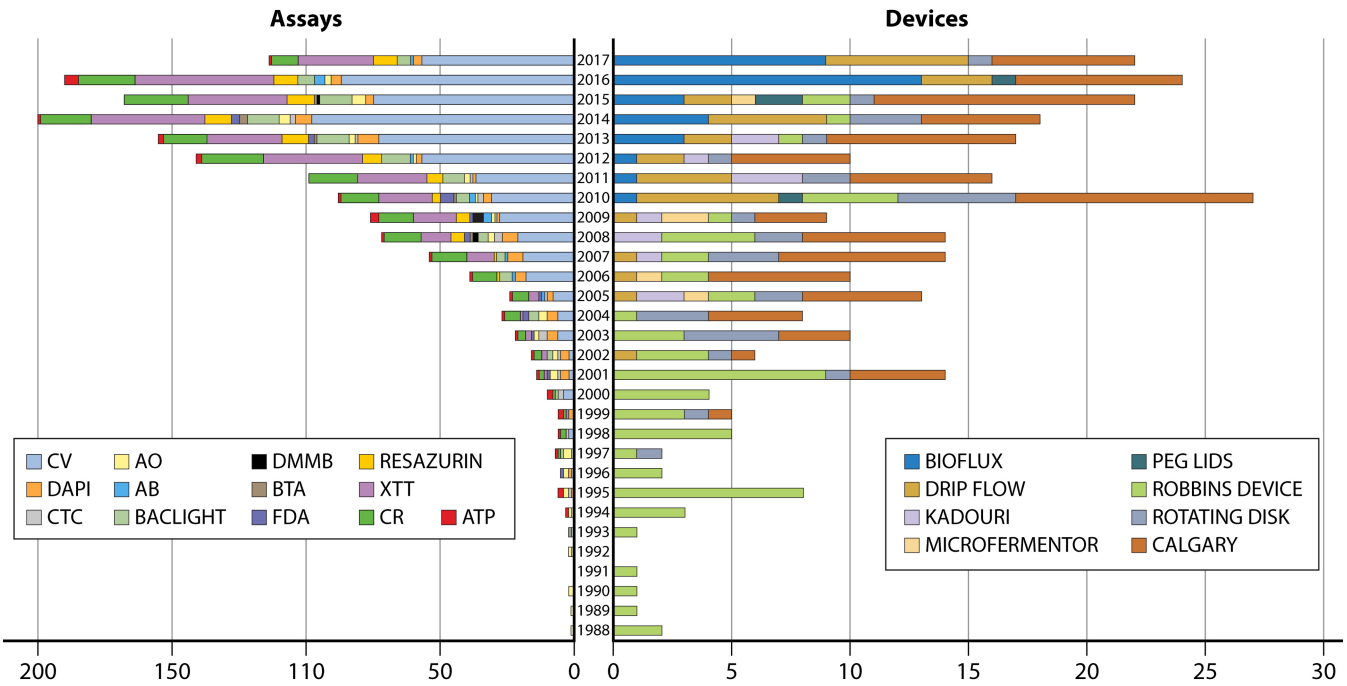


FIG 5 Illustrative quantitative and time-dependent representation of biofilm-based methodology publications for currently used devices and assays. CV, crystal violet; DAPI, 4′,6-diamidino-2-phenylindole; CTC, oxidized 5-cyano-2,3-ditolyl tetrazolium chloride; AO, acridine orange; AB, alamarBlue; DMMB, dimethylmethylene blue; BTA, biotimer assay; FDA, fluorescein diacetate; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; CR, Congo red.

without an abstract or in a language other than English and papers with irrelevant content were excluded from the search process.

What we deduce from the graphical representation of biofilm-based methodology publications is that the number of research articles for assays is higher than that for devices, with an average ratio of 7 assay publications to 3 device publications and a statistically significant difference ($P < 10^{-4}$). It is also evident that in the approximately 30-year timeline of the query, methodologies are irregularly distributed, indicating a lack of consistency and approval. Figure 5 reveals that (i) the peg lid CBD-MBEC and microfluidics-based devices stand out, with a dynamic presence and increasing rates of use; (ii) the drip-flow cell reactors and rotating-disk reactors might be occasional choices for biofilm growth; (iii) the Kadouri system has remained an interesting alternative in the past decade; (iv) CV, XTT, Congo red, Live/Dead BacLight, and resazurin assays show continuously increasing rates of use; and (v) DAPI appears to be a less popular yet solid choice that never acquired a dynamic rate similar to those of the five major assays for biofilm viability assessment. Another interesting observation is that significant development of different technologies for both assays and devices took place between 2008 and 2015 (up to 12 different assays and 7 different devices). Although an exponentially growing number of assays and devices were developed and applied, only a few have prevailed. More specifically, CV, XTT, and Congo red assays were the predominant methodologies among assays, while the Bioflux, Calgary, and drip-flow technologies dominated among devices over the past 6 years. This observation leads us to the conclusion that experimentation in developing novel methodologies in the biofilm research area is declining and that most of the methods have been abandoned by the research community.

Through implementation of the same search criteria, 4.9% of the publication fraction is dedicated to biofilm techniques over biofilm research (1,839/37,833 publications). This percentage is extremely low, taking into consideration the gap in studying multispecies biofilms under realistic environmental or clinical conditions. In order to strengthen our result that the percentage of publications regarding biofilm techniques

over biofilm research is low, we attempted to evaluate the publication fraction of another research area's methodological achievements. Genome editing is a large and promising area of research, leading the way toward exploring the potential of new developments by implementing a wide range of novel tools and techniques (395). Interestingly, the methodologies applied in the study of genome editing, until September 2017, amounted to 23.4% (2,355/10,062 publications) of publications, with the following keywords used as queries in PubMed searches: ("genome editing" or "chromosomal editing" or "genome inversions" or "chromosomal inversions" or "genome translocation" or "chromosomal translocation" or "structural variations" or "engineered nucleases" or "nucleotide repeats" or "DNA repeats") and ("double-strand breaks" or "DSBs" or "zinc-finger nuclease" or "ZFN" or "transcription activator-like effector nuclease" or "TALEN" or "clustered regularly interspaced short palindromic repeats" or "CRISPR" or "CRISPR-associated protein 9" or "Cas9" or "RNA-guided engineered nuclease" or "RGEN").

The field of biofilm research has proved that the establishment and definition of a "perfect method" comprise a rather precarious generalization. Taking for granted the uniqueness of bacterial biofilms as vivid microcommunities, the approach of a "one-size-fits-all" methodology would abolish any attempt at a thorough understanding. Pluralism in available methodologies is instrumental in order to address basic questions about biofilm formation, structure, adherence, physiology, kinetics, and interactions with the host.

State-of-the-Art Methodologies in Biofilm Investigation

The majority of information that constitutes the current knowledge on biofilm formation patterns is based on *in vitro* studies. Combined technologies simulate biofilm growth and formation conditions. However, studying biofilms in the lab provides information which differs significantly from our experience in clinical practice. Such an observation defines the need for further experimental accuracy in order to link *in vitro* and *in vivo* outcomes. The host habitat, regarding immunity parameters as well as the components of the human body (tissues [acting as adherence surfaces] and body fluids, such as urine and blood), constitutes a challenging landscape for bacterial growth which differs from experimental conditions (396). Some state-of-the-art compositions and devices have already entered the market over the last decade (Table 4).

Biofilm growth detection based on molecular probes or staining agents enables direct observation, quantification, and topographical mapping of bacterial growth *in vivo* or on medical tool surfaces (397–402). A number of adherence assays exploit biofilm properties and detect biofilm formation and structure (403, 404). Indwelling medical devices carrying color change indicators integrated in substrates prone to degradation in the presence of bacteria mediate detection when applied *in vivo* (405, 406). Immunoassays and enzymatic methods certify biofilm presence and assist in quantification (407, 408). Other *in vivo* theranostic devices employ imaging and sensing tools enabling biofilm "fingerprint" detection through data collection and analysis of thickness, growth speed, and physicochemical properties (409–412).

Combinatorial application of older and conventional methodologies for both *in vitro* and *in vivo* biofilm investigation constitutes a significant resource for the launch of cutting-edge tools. Enhancement of techniques by use of sensing modules, bioinformatics, and the inspirational introduction of biomaterials in surface construction have provided alternative insights into biofilm research. Where lies the difference between research lab methodologies and inventions? What is the clinical significance of the innovation landscape? Sum-of-the-parts comparative analysis gives answers regarding the differences between conventional methodologies and innovative tools. Commercially exploited staining methods differ from those applied in the academic or clinical lab, including a wider variety of biocompatible dyes offering a speedy and easy way to detect results. In-host commercial theranostic devices allow real-time information on the applicability and success rate of the technique right from the bedside. Finally, computational and biosensing methods embedded in daily routines, even adjusted to

TABLE 4 Innovative platforms for biofilm evaluation[a]

Patent no.	Method	Description	Reference(s)
US 8399649 B2	Molecular probes for biofilm-related protein expression	*mucE* and *algW* gene expression determination facilitates monitoring of biofilm formation initiation in human specimens and indwelling devices	397
US 20120322048 A1	Topographical detection on living tissues	Fluorescent staining agents (i.e., alcian blue and ruthenium red) topically react with extracellular matrix biofilm components found on wounds	398
EP 2537601 A1, EP 2634260 A1	Colorimetric detection assay	The detection kit contains a staining solution (i.e., aqueous solution of Coomassie blue, crystal violet, safranin, ruthenium red, rhodamine, or erythionine) and a rinsing solution (i.e., oxidizing or chelating agent) which identify the presence of biofilms on surfaces upon application	399, 400
US 7955818 B2	Microbial culture viscosity measurement	The motion of a charged particle driven by electrical, magnetic, or electromagnetic field application is indicative of biofilm presence in a microbial culture	401
US 20080176265 A1	Potassium permanganate staining solution	Potassium permanganate is applied as a slime matrix staining agent or contrast enhancer for biofilm microscopic visualization or quantification	402
US 20060275847 A1	Combinatorial automated structure analysis	Electromagnetic radiation and CLSM assays of fluorescent biofilm moieties are combined with computational image analysis to provide a multiplanar structure determination	403
US 20030177819 A1	Tools to validate clinical sterility	A flowchart of metabolic, imaging, and immunological assays can be used to certify the absence of biofilm formation on medical surfaces and devices (respirometry, Live/Dead staining, CLSM, SEM, AFM, FISH probing, ELISA)	404
US 20140356901 A1	Urine-detectable colorimetric assay	Indwelling device colonization is detected by the degradation of a polymeric substrate doped with a blood-soluble and urine-passable dye (i.e., methylene blue, β-carotene, rifampin, Evan's blue, indocyanine green, or betanin)	405
US 20140352602 A1	Hydration/pH change indicator	Color change of a moisture indicator identifies biofilm presence on catheter surfaces	406
US 20100285496 A1	Lateral flow immunoassay	Labeled antibodies certify the presence of a biofilm-related target analyte	407
EP 1067385 B1	LAL assay	The reaction of LAL with bacterial endotoxins or lipopolysaccharides mediates surface-associated biofilm load quantification	408
WO 2014052449 A1	Photoacoustic flow cytometer	A photoacoustic flow cytometer is applied to blood and/or lymphatic circulation, and photoacoustic pulse analysis enables biofilm quantification	409
US 8697375 B2	MRI-based diagnosis	MRI scanning detects probes that are selectively bound to biofilm found either on mammalian tissues or on indwelling medical devices	410
US 8233957 B2	Electrochemical sensing system	pH and impedance sensor modules placed onto catheter surfaces calculate biofilm thickness, speed of growth, and chemical properties	411
US 20120295216 A1	Diagnostic ultrasonic toothbrush	An ultrasonic sensor detects biofilm thickness by temp, oximetry, proximity, and pH measurements	

[a]CLSM, confocal laser scanning microscopy; SEM, scanning electron microscopy; AFM, atomic force microscopy; FISH, fluorescence *in situ* hybridization; ELISA, enzyme-linked immunosorbent assay; LAL, *Limulus* amoebocyte lysate; MRI, magnetic resonance imaging.

the size of a toothbrush, set the road for the development of devices that will eventually enter clinical settings.

Extending beyond Commonplace Platforms

Quartz tuning forks (QTFs) have been applied for *P. aeruginosa* biofilm growth monitoring and (bio)sensing (28, 413). The adhesion dynamics that determine biofilm development are monitored by use of piezoelectric tuning forks. QTFs also enable biomass growth detection as well as antibiotic testing and determination of nanomechanical surface properties (414).

A polyurethane-coated, magnetostrictive, ribbon-like sensor as part of a flow system connected to a bioreactor has been used to wirelessly monitor *P. aeruginosa* biofilm formation with the aid of magnetic field telemetry (415). Electromagnetic (EM) wave sensors offer a reliable, real-time, cost-effective methodological tool for *in situ P. aeruginosa* growth analysis. EM wave sensors mounted on a polymethyl-methacrylate microfluidic cell structure contributed to the early detection of biofilm growth upon excitation at microwave frequencies (416).

Chronicity due to AMR stems from the lack of detection methodologies for clinically relevant biofilms formed at an early stage. A shift in this reality would potentially support better treatment options. This concept was the foundation for development of a coupled optical and acoustic imaging technology that noninvasively detects and quantifies biofilm biomass. Ligand-targeted UCAs assessed the ability of *S. aureus* to form communities *in vitro* (386). High-frequency scanning acoustic microscopy (SAM) coupled with UCAs facilitated ultrasonic imaging and quantification of the mechano-elastic biofilm properties. This method was proven to be efficient, though the similarity of biofilm acoustic impedance (1.9 MRayl) and that of human soft tissues (1.35 to 1.85 MRayl) poses a serious limitation to *in vivo* imaging.

Bacterial aggregates require specific spatial distribution and environmental parameters to develop physicochemical properties that confer clinically relevant phenotypes (virulence factor production, AMR, and biofilm formation) contributing to strain-specific pathogenicity (417–419). The technical drawback of monitoring the behavior of these populations due to their small size (10^1 to 10^5 cells) was recently overcome by use of a laser-based lithographic technology that provides microscopic printing of the 3D geometry of the bacterial aggregates, along with scanning electrochemical microscopy (SECM) enabling redox-active, biofilm-derived small-molecule quantification (for example, that of pyocyanin from *P. aeruginosa*) (420–422). Coupling of these two analytical tools achieves real-time quantitative monitoring of bacterial aggregate interplay and assesses the impacts of spatial organization and chemical signaling on sociomicrobiology.

Extending the use of 3D printing technologies, a novel material consisting of the broad-spectrum antibiotic nitrofurantoin and the biodegradable polymer poly(L-lactic acid) was manufactured to mimic catheters. The 3D-printed disks exhibited >85% biofilm inhibition; therefore, an antimicrobial option has emerged for medical device coatings (423). One of the most recent setups for real-time *E. coli* and *P. aeruginosa* biofilm biomass growth evaluation was achieved through a sensing microsystem in which a microfluidic flow reactor employs a surface acoustic wave (SAW) sensor and electrodes constituting a source for current signals. This electric field is coupled with the use of gentamicin, and therefore this integrated sensing platform serves for biofilm growth monitoring and biofilm elimination (424). A novel impedimetric sensing system based on the interdigitated microelectrode microsystem envisions paving the way for the development of smart biosensors for rapid implant-associated biofilm identification and removal. In short, this microsystem allows label-free *E. coli* biofilm growth detection in microfluidic channels by evaluating the fractional relative change in real time as well as monitoring the threshold-activated bioelectric effect on the *in situ* treatment process (425). A custom-made surface plasmon resonance (SPR) biosensor was recently applied for *E. coli* biofilm formation investigation on gold-plated glass disks. In particular, the angle-based SPR biosensor allowed real-time capture of the SPR curve as well as calculation of the refractive index change, thus offering a complete picture of the biofilm formation cycle (426).

CONCLUDING REMARKS

It is evident that most experimental biofilm procedures provide descriptive and quantitative information, but combining tools and methods unravels more pieces of the puzzle. Most of the conclusions obtained here are derived from *in vitro* studies, yet their relevance to the processes and methodologies occurring *in vivo* are a subject for further consideration and experimentation. Recalcitrant and persistent biofilm-

associated diseases have raised the need for new therapeutic approaches and methods for reliably culturing mature biofilms and evaluating their chemical, structural, and physiological characteristics.

The lack of consistent and robust animal biofilm models is perhaps the most critical element missing from the equation. It appears that engineering-driven approaches give and will continue to give methods and tools transcending the current norm and status of biofilm analysis. This pool is still in the periphery of clinical knowledge. Thus, the fundamental questions remain partially answered. That is, how do we detect biofilm formation at the bedside, and what is the best course of action for eradication? Commercially available kits and the wealth of research information can educate but barely provide comprehensive and articulated answers. A more compelling set of answers may arise when the intersection of clinical and engineering approaches becomes relevant.

Advances regarding the microbiome and unculturable bacteria designate the imperative need for reliable tools for thorough investigation of host-biofilm interactions. Consequently, the establishment of robust biofilm susceptibility assays also remains a challenge for clinical entities. Information stemming from combinatorial assays may provide comprehensive and critical insights into any given biofilm-associated clinical query.

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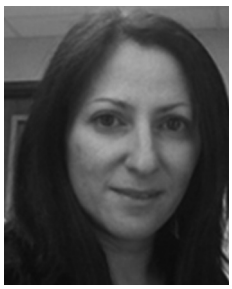

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