BBA Advances 7 (2025) 100133

Contents lists available at ScienceDirect

BBA Advances

journal homepage: www.sciencedirect.com/journal/bba-advances

# Capturing the micro-communities: Insights into biogenesis and architecture of bacterial biofilms

Harshita Agarwal [a], Bharat Gurnani [b], Bhumika Pippal [a], Neha Jain [a,*]

[a] Department of Bioscience and Bioengineering, Indian Institute of Technology Jodhpur, NH 65, Nagaur Road, Karwar, Rajasthan 342037, India
[b] Centre of Excellence-AyurTech, Indian Institute of Technology Jodhpur, NH 65, Nagaur Road, Karwar, Rajasthan 342037, India

ARTICLE INFO

*Keywords:*
Biofilm
Matrix
Components
Biofilm biogenesis
Biofilm-imaging

ABSTRACT

Biofilm is an assemblage of microorganisms embedded within the extracellular matrix that provides mechanical stability, nutrient absorption, antimicrobial resistance, cell-cell interactions, and defence against host immune system. Various biomolecules such as lipids, carbohydrates, protein polymers (amyloid), and eDNA are present in the matrix playing significant role in determining the distinctive properties of biofilm. The formation of biofilms contributes to resistance against antimicrobial therapy in most of the human infections and exacerbates existing diseases. Therefore, this field requires several state-of-the-art techniques to fully understand the 3-D organization of biofilms, their cell behaviour and responses to pharmaceutical treatments. Here, we explore the assembly and regulation of biofilm biogenesis in the context of matrix components and highlight the significance of high-resolution imaging and analysing techniques for monitoring complex biofilm architecture. Our review also emphasizes the novelty and advancements in techniques to visualise biofilm structure and composition, providing valuable insights to understand biofilm-related infections.

## 1. Introduction

Bacteria prefer to reside as a community beneficial for survival and propagation under diverse environmental conditions [1]. These communities, known as biofilms, consist of aggregates of cells and can be observed on biotic and abiotic surfaces [1]. Bacterial cells within a biofilm (persistor) are more adaptive and resilient than free-living (planktonic) cells [1]. Biofilm facilitates cross-talk between cells and the surrounding environment. The intercellular interactions enable bacteria to synchronize gene expression for adequate resource management [2]. Within the biofilm, bacterial cells exhibit a high degree of tolerance towards environmental insults, host immune response, antibiotics, disinfectants, shear forces, and other adverse conditions [2].

Bacterial cells within biofilms are protected by a self-produced hydrated polymer matrix that holds nutrients and water [2]. The matrix components called extracellular polymeric substances (EPS) include extracellular nucleic acid (eDNA), polysaccharides, and protein polymers [3]. The dynamic nature of EPS allows the biofilm to be considered a hydrogel that manifests viscoelastic behavior, which helps the cells tolerate mechanical stress [4]. The switch from planktonic life to sessile biofilm formation is a well-coordinated stepwise process governed by modulation of gene expression and regulatory pathways [4]. The biological, physical, and biochemical features of biofilms promote horizontal gene transfer, a major pathway to acquire antimicrobial resistance (AMR) genes [5]. Impermeability of antimicrobial molecules in the biofilm, inactivation of drug molecules, decreased effective drug concentration before reaching the target site, efflux systems, restricted diffusion, and persistent cells all contribute to biofilm antibiotic resistance [6].

Besides AMR, biofilms have further potential to cause chronic infections that persist for extended periods, making the process of repair and healing more challenging. The biofilms in incisions and wounds may culminate in various complications, posing a serious risk to the individual [7]. Recently, biofilms have been found to induce an inflammatory response that injures the tissues and delays the healing process [8]. In chronic infections, biofilms protect bacterial cells from the human defence mechanism and other antimicrobial drugs [7]. Biofilms also modulate the host environment, making it favorable for colonizing other pathogenic bacteria. For example, *Streptococcus mutans* can change the local surroundings to be ECM-rich and have low pH, accommodating other aciduric and acidogenic species to thrive and contributing to the development of dental caries [9]. A better interpretation of composition

* Corresponding author.
*E-mail address:* njain@iitj.ac.in (N. Jain).

https://doi.org/10.1016/j.bbadva.2024.100133

Available online 21 December 2024
2667-1603/© 2024 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

and matrix formation may contribute to developing drugs targeting biofilms and related infections. In biofilm research, several microscopy and spectroscopy techniques have been utilized, which helps us advance our understanding of their structure [10]. Combinations and variations in techniques have emerged as efficient tools to observe, locate, and quantify microorganisms, components, and genes inside biofilms.

This review illustrates detailed insights into major matrix components that provide enormous strength to the biofilm and contribute to the severity of biofilm-related diseases. We also shed light on the powerful techniques deployed to image and analyse biofilms. These methodologies offer details into the quantity, interactions, and mobility of different molecules in the biofilms and the characteristics of the surrounding microenvironment. These methods can also be used to investigate how physicochemical characteristics vary in real time due to the addition of external agents during the development of biofilms. We believe that compositional and structural information on major matrix components such as amyloids and eDNA gleaned from state-of-the-art spectroscopy and microscopy modalities will help the field to identify novel targets for therapeutic interventions to combat biofilm-related chronic infections.

## 2. Process of biofilm formation

Biofilm biogenesis is a complex, multifaceted, and dynamic process [2]. As shown in Fig. 1, biofilm development is a stepwise process that includes initiation and attachment, followed by maturation and dispersion. Here, we provide an overview of the different stages of biofilm development governed by adaptive responses.

### 2.1. Initiation

The initial attachment of cells to the surface strongly depends on the hydrophobic-hydrophilic properties of the adhering surface [11]. Factors such as temperature, pressure, bacterial orientation, surface area, and properties influence the initial attachment [12]. Surface structures such as flagella, fimbriae, type IV pili, proteins, and polysaccharides play a crucial role in the initial attachment [13]. While flagella facilitate the initial attachment of cells with the surface, the twitching motility of type IV pili aids cells in aggregating and forming microcolonies [12]. The matrix proteins such as fibronectin, fibrinogen, and vitronectin are known to facilitate biofilm initiation during human infections [4]. Hydrolytic enzymes called autolysins also facilitate initial attachment *via* noncovalent interactions. The initiation can be categorized into two stages: reversible attachment and irreversible attachment. In reversible attachment, bacterial cells attach to surfaces *via* nonspecific van der Waal forces, steric and electrostatic interactions [4]. The cells adhere and detaches multiple times until stable contact with the surface is established [3]. Once the cells are irreversibly bound to the surface, they become remarkably tolerant to physical and chemical shear forces [3]. The irreversible attachment induces specific gene expression that drives biofilm maturation [4]. In *E. coli,* after irreversible attachment, gene expression regulation is markedly affected by many variables. A key example pertains to the *csgD* gene, which is essential for biofilm development. The expression of *csgD* is favorably modulated by global regulators, including IHF (Integration Host Factor) and Fis (Factor for Inversion Stimulation), which augment its promoter activity. This control results in enhanced synthesis of fimbriae and cellulose, which are the critical constituents of the biofilm matrix [14].

### 2.2. Maturation

The initial attachment is followed by the rapid multiplication of cells, resulting in increased biomass and microcolony formation, where cells get arranged according to their metabolism and tolerance to the availability of oxygen [15]. Cell proximity in a microcolony provides a suitable niche for exchanging nutrients, metabolites, gases, signaling molecules, and genes [13]. Regulation of specific genes within microcolonies triggers the production of extracellular polymeric substances (EPS) in the matrix, followed by the formation of water channels and fluid-filled voids [16]. The channels act as circulatory systems facilitating the distribution of nutrients, enzymes, metabolites, and waste removal within the biofilm [16]. The biofilm maturation depends on a delicate balance between adhesive factors such as proteins, eDNA, and lipids and disruptive factors like proteases and nucleases that mediate rearrangement and establish cells in the extracellular matrix. Within the mature biofilm, the bacterial cells share and exchange resources that contribute to the dynamic architecture of the biofilm and provide a suitable niche for the cells to survive and become more tolerant to adverse conditions [13].

### 2.3. Dispersion

Lack of nutrients and surface for further growth induces dispersion or detachment of mature biofilms [17]. The factors contributing to dispersion include oxygen fluctuations, nutrient depletion, temperature variation, increase in toxic by-products, cell density, stress conditions, and metabolite accumulation [17]. The sensory systems in bacteria monitor the level of small molecules as cues for the altered environment and regulate gene expression accordingly, leading to dispersion [18]. During dispersal, there is an up-regulation of genes related to cell

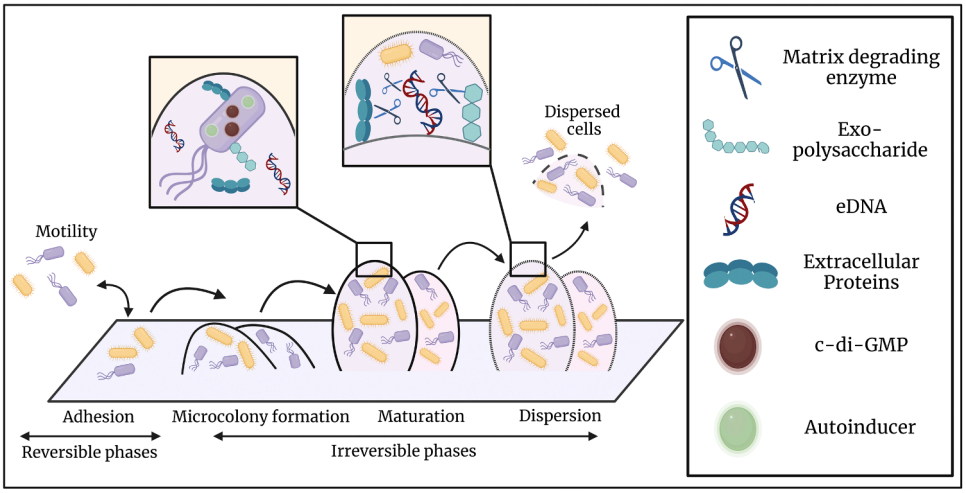


**Fig. 1.** Process of biofilm formation on a suitable surface.

motility and EPS breakdown, whereas genes associated with EPS generation are down-regulated [17]. Several regulatory mechanisms, such as quorum sensing, nucleotide-based secondary messenger signaling, and small RNA regulatory pathways, play a pivotal role in the dispersal [17]. Enzymes that degrade matrix components, molecules that reduce surface tension, and form cavities due to cell lysis promote biofilm dispersal [19]. Similar to formation, dispersal is a complicated step in the biofilm life cycle that requires coordinated efforts from cells within the biofilm [17]. During the dispersion event in *Staphylococcus aureus* biofilms, several critical genes are activated to promote detachment and transition to a planktonic form. The *agr* system, a quorum-sensing regulator, amplifies the expression of virulence factors and enzymes that facilitate biofilm dispersal. The *nuc* gene, which encodes nuclease, is activated to destroy e-DNA within the biofilm matrix. Furthermore, *clpC* (required for protein quality regulation), and the *lrgAB* operon (responsible for regulating cell lysis) are elevated. Many genes collectively facilitate efficient biofilm dispersal, hence enhancing colonization and evasion of host defences [20]. Also, dispersal provides a unique opportunity for the bacterial cells to re-establish at different sites during infections, thereby increasing the chances of survival [16].

## 3. Role of signaling pathways in biofilm development

Biofilm development relies on signaling pathways to respond to external environmental cues [21]. The primary regulators in signaling pathways of bacterial biofilm include quorum-sensing molecules [21], secondary messenger such as cyclic diguanosine monophosphate (c-di-GMP) [22] and small RNAs (sRNAs) [23].

### 3.1. Quorum sensing

Quorum sensing (QS) is a cell density-based bacterial intercellular communication mechanism. The process occurs via small, self-generated, and diffusible signalling molecules called autoinducers [21]. Autoinducers are released at a low cell density and accumulate in the surrounding environment [24]. Once the bacterial population surpasses a certain threshold, autoinducers attach to particular receptor proteins to control the activity of genes related to virulence and biofilm formation [24]. Gram-negative bacteria utilize N-acyl-l-homoserine lactones (AHLs) as autoinducers, while gram-positive bacteria employ small peptides. Autoinducer-2 (AI-2) is a QS molecule present in almost all bacterial species [24]. QS significantly governs social interactions within the biofilm like cooperation and competition among the resident bacterial species. It also plays a crucial role in the maturation and dispersion of biofilms [25]. QS systems function synergistically to regulate the expression of many genes vital for biofilm formation, maintenance, and dissemination. For instance, in *P. aeruginosa* primary genes involved in QS that regulate biofilm formation are part of two major systems: the las system and the rhl system. The *lasI* gene encodes the enzyme responsible for synthesizing the N-(3-oxododecanoyl) AHL and *rhl* system is crucial for N-butyryl AHL [26]. QS offers a potential therapeutic target for bacterial diseases, and the use of QS inhibitors to combat viruses could effectively supplement current medications. Quorum quenching can reduce microbial pathogenicity and enhance antibiotic susceptibility in microbial biofilms, making it an attractive strategy for developing new drugs in the fight against microbial diseases [25].

### 3.2. Small non-coding RNA (sRNA)

sRNA, also known as short-bacterial non-protein coding RNA (npcRNA), is a group of small RNA molecules, usually 50–500 nucleotides in length, that are unable to translate into proteins [27]. These play a significant role in gene expression, stability, and biofilm development [28]. sRNAs employ two different ways to control the formation of biofilms: base pairing with other RNAs and protein binding [23]. By regulating various physiological responses in bacteria, sRNAs form an important part in the regulatory network for biofilm formation [28]. In *P. aeruginosa,* rsmY and rsmZ are two best-known examples of sRNA. Increased expression of rsmY and rsmZ enhances the initial attachment of bacteria on the abiotic surface; however, high levels of sRNAs can slow down biofilm development [23]. *V. cholerae* has four redundant sRNAs termed Qrr1–4 that regulate quorum-sensing during biofilm formation [29].

### 3.3. Cyclic diguanosine monophosphate (c-di-GMP)

The c-di-GMP is an intracellular signalling molecule involved in "lifestyle transition" in many bacteria [22,30]. The correlation between levels of c-di-GMP and biofilm formation is observed in various bacterial species, including *E. coli, P. aeruginosa, Salmonella enterica,* and *V. cholerae* [31]. The factors modulated by c-di-GMP in the biofilm life cycle include surface adhesion, biosynthesis of exopolysaccharide matrix, secretion of eDNA, secondary metabolite production, antimicrobial resistance, and biofilm dispersion [22]. Moreover, the correlation between c-di-GMP concentrations and biofilm matrix formation is essential for biofilm development. Elevated intracellular concentrations of c-di-GMP facilitate biofilm development by augmenting the production of exopolysaccharides, adhesins, and other matrix constituents, which are crucial for the structural integrity and stability of the biofilm. In contrast, diminished levels of c-di-GMP promote a planktonic lifestyle by reducing matrix formation and increasing bacterial mobility. This dynamic control enables bacteria to shift efficiently between sessile and motile phases in response to environmental stimuli, therefore affecting survival and pathogenicity [31]. The c-di-GMP is used as a checkpoint to proceed with biofilm formation until bacteria have acquired a stable biofilm lifestyle [22,32].

## 4. Matrix: The dynamic wall of biofilm

Cells in a biofilm are held together and with the substrate by a physical scaffold of extracellular matrix (ECM) that provides structural support and framework to the three-dimensional organization of biofilms. The complexity of matrix biopolymers and the difficulties in researching ECM have led to them being called "the dark matter of biofilms". The term "matrixome" is introduced to provide in-depth information on matrix composition, its diverse functions, and its role in virulence during infections. The matrix serves as a reprocessing and reservoir hub that holds lysed cell components such as DNA, intracellular proteins, and extracellular enzymes [19]. Extra Polymeric Substances (EPS) are the major component of the matrix that makes a stable scaffold for biofilm architecture and provides a desirable niche for microcolony formation. The matrix components such as extracellular DNA (eDNA), proteins and polysaccharides enable bridging the gap between cells, development of high cell densities, temporary immobilization of bacterial populations, and cell-cell recognition [19]. Fig. 2 depicts various components of EPS that constitute the matrix. The following subsections will cover the major components of EPS and their role in biofilms.

### 4.1. Water

Water accounts for 97% of the bulk of the biofilm matrix, making it by far the most abundant component [19]. The EPS matrix creates a highly moist microenvironment that dries relatively slower than its surroundings, protecting biofilm cells from dehydration and variations in osmotic potential. Desiccation is one of the environmental factors that EPS offers significant advantages to biofilm [33]. The EPS matrix can sequester polar and apolar molecules from the aqueous phase by acting as a molecular sieve [2].

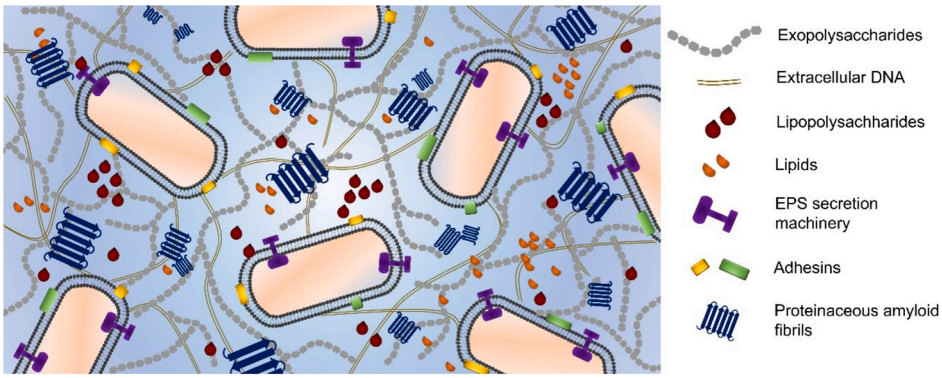


**Fig. 2.** Graphical representation of extracellular polymeric substances (EPS) composition in the biofilm matrix.

### 4.2. *Extracellular proteins and functional amyloids*

The proteinaceous biofilm components comprise adhesins, extracellular proteins, outer membrane vesical proteins, and appendages (flagella and pili) [19]. Structural and localization analyses reveal the contribution of extracellular proteins in surface attachment, matrix organization, matrix degradation *via* enzymatic activity, and interaction with exopolysaccharide and nucleic acid [19]. Non-enzymatic proteins such as lectins bind to polysaccharides and establish a connection between the bacterial cell surface and matrix. Matrix enzymes break down matrix components that help in the dispersion and conversion of biopolymers into carbon and energy-rich products. Surface adhesins such as Bap and SasG and fibronectin-binding proteins play a significant role in cellular adhesion and promote biofilm formation in *Staphylococcus aureus* [34]. In many biofilm-forming bacterial species, extracellular proteins can form a self-polymerized and highly organized fibril structure called amyloids [35]. These amyloids help bacteria in surface attachment, enhanced virulence, matrix architecture, interaction with exopolysaccharides and eDNA and host interaction, survival, and fight antimicrobials, thus called Functional Bacterial Amyloids (FuBA) [35,36]. FuBA have distinct structural and biochemical features but are comparable to pathogenic amyloids [36]. Primary structures of FuBA are optimized for amyloid formation by controlled assembly of subunits into classical cross-β-sheet or a unique cross-α-fold structure [36]. Curli was the first discovered and characterized FuBA, responsible for cell aggregation, adhesion, cell interaction, and biofilm formation in *E. coli and Salmonella* [37]. Curli also plays a key role in host invasion and interaction with host proteins and immunity factors and can shield bacteria from immunological responses by sequestering antimicrobial peptides and blocking the traditional mechanism of complement cascade activation [37]. Pseudomonas also forms genetically distinct but morphologically similar functional amyloids referred to as Fap amyloids. Fap amyloid fibrils play a significant role in biofilm formation, contributing to increased hydrophobicity, reversible binding to signaling molecules, and enhancing biofilm stiffness [38,39]. FapC fibril-producing cells are known to exhibit improved retention of iron-chelating metabolites and help sequester specific extracellular metabolites, quorum-sensing molecules, and redox mediators that move across the matrix between cells [40]. The soil bacterium *B. subtilis* biofilm has TasA and TapA as the amyloid components that play a significant role in biofilm integrity and matrix. TasA amyloid fibrils attach to exopolysaccharides during multispecies biofilm formation, facilitating interspecies interactions [41]. TasA also maintains secondary metabolite production and activity, regulates membrane dynamics, and enhances stress tolerance [41]. *S. aureus* biofilm matrix has major amyloidogenic α-helical amphipathic peptides called Phenol Soluble Modulins (PSMs) that contribute to its stability, maturation, integrity, and resistance to antibiotics [42,43]. Biofilms are further stabilized by the polymerization of PSM1 in S. aureus strains that permit autolysis and release of eDNA [44]. Functional amyloids from bacterial biofilms, such as Esp from *E. faecalis*, Aap from *S. epidermidis*, and AM-1in *S. silvestris* are recently discovered [36,45,46]. FuBA is a crucial structural and functional component within most biofilms.

### 4.3. *Exopolysaccharides*

Extracellular polymers of carbohydrates or exopolysaccharides constitute a significant fraction of the biofilm matrix and play a critical role in cell adherence to the surface, capsule formation, protection from environmental stress, and bacterial virulence [3]. They form a dense network that imparts a three-dimensional structure and confers mechanical stability to the biofilm matrix [3]. Exopolysaccharides may be synthesized either extracellularly or intracellularly [3]. The physical and chemical properties of the matrix largely depend on the type of exopolysaccharides produced [3]. Diverse microbial species produce a wide range of exopolysaccharides that vary in composition, physicochemical properties, and molecular ratios [3]. They are indispensable for many bacteria in biofilm formation; however, in polymicrobial communities with multispecies, only a few species may synthesize exopolysaccharides and contribute to matrix formation [3]. Most exopolysaccharides are polyanionic due to uronic acid or ketal-linked pyruvates such as alginate, colanic acid, and xanthan [3]. Many bacteria produce polysaccharide intercellular adhesin (PIA), a poly-N-acetylglucosamine (PNAG) necessary for intercellular adhesion, biofilm-support, protection from antibiotics, and structural integrity of the biofilm matrix [47].

The matrix of *P. aeruginosa* contains three distinct exopolysaccharides: alginate, Psl, and Pel [48]. Alginate is a polymer of mannuronic acid and guluronic acid, which plays a critical role in the spatial organization of matrix, antibiotic resistance, and protection from host immune response and antimicrobial peptides [49]. Pel, a cationic exopolysaccharide composed of partially acetylated N-acetylgalactosamine and N-acetylglucosamine, crosslinks with eDNA to facilitate initial attachment and provide structural integrity to the matrix [48]. Cellulose, a polysaccharide of β−1,4 linked d-glucose, is a major matrix component in *E. coli* biofilm [50]. It interacts with extracellular protein fibrils during biofilm formation in *E. coli* [50]. The high tensile strength of cellulose provides structure to the matrix, aids cell aggregation, and protects from the host immune response [50]. Vibrio exopolysaccharide (VPS), composed of glucose and galactose, with traces of N-acetylglucosamine, mannose, and xylose, is the main biofilm matrix component in *Vibrio cholerae* essential for biofilm formation, attachment of cells, and accumulation of matrix proteins [51]. Most of the exopolysaccharides including cellulose, alginate, Pel polysaccharide and, PNAG, have been shown to play an important role in bacterial biofilm formation and have indispensable role in biofilm virulence and resistance, emphasizing their importance as potential targets for disrupting biofilm formation and treating persistent infections [52].

### 4.4. Extracellular vesicles

Extracellular vesicles (EVs), such as exosomes and microvesicles, are produced to facilitate cell interactions and eliminate undesired cellular contents. EVs discharged into the extracellular environment, within biofilms, can travel to body fluids, cells, and distant tissues. They can carry a range of functional or biological cargo such as RNA, eDNA protein, and lipid to nearby and distant cells. These functional cargo may alter the state of the recipient cells, influencing both physiological and pathological processes [53]. They are a significant but often overlooked aspect of the biofilm matrix, primarily found in Gram-negative and mixed-species bacterial biofilms. EVs could be seen in mechanically disrupted biofilms and can be isolated with matrix components, confirming their inevitable presence and distribution in the biofilm matrix [54]. Furthermore, EVs derived from planktonic bacteria and biofilms were found to differ both quantitatively and qualitatively and exhibited functions such as antibiotic binding and enzymatic activity [54]. Another study by Tashiro et al. reported that extracellular membrane vesicles containing enzymes help in matrix degradation in Gram-negative bacteria [55]. EVs serve structural, virulence, and pathogenic roles in the EPS of several bacterial species. The analysis of the composition and biogenesis of EV may help identify elements that may be (i) the target of novel medications that prevent the formation of biofilms, (ii) vaccine candidates, or (iii) biomarkers for the detection of bacterial infections [56].

### 4.5. Lipids

Lipids are one of the main components of biofilms. The composition of fatty acyl chains in lipid determines its biophysical characteristics, thus affecting its biological role in biofilms. It plays a significant role in structure, energy storage and signalling within the biofilms [57]. Lipid rafts are also involved in biofilm formation for instance phosphatidylinositol is precursor for lipid rafts in *C. albicans* biofilms. Studies that analyze lipidomes (total lipid content), suggest that the lipidome changes with the age of the biofilm [58]. Pathogenic bacteria, including *Salmonella Typhimurium, S. aureus, Listeria monocytogenes, P. aeruginosa,* were cultured in planktonic or biofilm states to investigate membrane fatty acid composition. It was found that the proportion of saturated fatty acids was higher in biofilm cells [59]. Lipids help microbes to survive in extreme conditions as lipids were also reported in the matrix of yeasts, fungi, sulfate-reducing bacteria, and mycobacteria in activated sludge. For instance, *Thiobacillus ferrooxidans* and *Serratia marcescens* produce lipids with surface-active properties that facilitate microbial adhesion to pyrite surfaces [60]. As an adaptive stress response, lipid in biofilm cells enables bacteria to control exchanges, conserve energy, and persist [59]. The remodeling of lipids in biofilms may explain bacterial resistance to the effects of biocides. Lipids play a crucial role in biofilm maturation and dispersion. Cis-2-decanoic acid (CDA) is essential for biofilm dispersal in *Pseudomonas* and several other bacterial species such as *E. coli, K. pneumonia*e, *Proteus mirabilis, S. pyogenes, B. subtilis*, and *S. aureus* [61].

### 4.6. Extracellular DNA (eDNA)

Initially appeared to be a side product of bacterial cell lysis, extracellular DNA (eDNA) was soon recognised as an essential and integral component of the biofilm matrix and life cycle [62]. eDNA contributes to the structural and functional properties of the biofilm matrix [62,63]. It protects cells from antimicrobial agents and hosts immune responses [63]. It is widespread in the environment and can be derived from different species with abundance depending on the origin [63]. In multispecies biofilm formation, low DNA producers influence the amount of eDNA produced in the environment *via* competitive and ecological interactions. The charge, length, chemical nature, and lattice-like structure of eDNA contribute to biofilm architecture [64]. It facilitates adhesion, enhances structural and mechanical integrity, and provides stability to biofilms *via* interactions with other matrix components [64]. Understanding the role of eDNA in biofilm can make eDNA a lucrative target for controlling biofilm-related infections. Typically, the genesis of eDNA involves two distinct pathways: the lysis-dependent pathway and the lysis-independent pathway. The lysis-dependent process induces cell lysis, carried on by lethal chemicals, including bacterial endolysin, prophage virulence factors, and antibiotics. In *P. aeruginosa* prophage endolysin can drive eDNA release via cell lysis [65]. Membrane vesicles (MVs) can also produce eDNA via a lysis-independent process. The eDNA in *Streptococcus mutans* is produced from MVs, which is essential for developing biofilms [66]. Recent reports suggest that the major source of eDNA in biofilm is neutrophil extracellular traps (NETs), which are highly organized biofilm components made up of protein and DNA and the sticky matrix surrounding the cell. Various gene regulation mechanisms mediate the release of genomic DNA by lysis of bacterial cells [67]. The eDNA release in *P. aeruginosa* is mediated by quorum sensing (QS) dependent and independent pathways [65]. The basal level of eDNA is released by the QS-independent pathway. However, the QS-dependent pathway based on acyl-homoserine lactone (AHL) molecules and quinolone signaling molecules contribute to enhanced levels of eDNA during the late-log phase of bacterial growth [65]. The intriguing study in *Staphylococcus epidermidis* demonstrated that activation of AtlE (an autolysin protein) results in the lysis of a subpopulation of cells, leading to the release of eDNA [44]. The deletion of *atlE* abolishes eDNA in the biofilm matrix [44]. The major contributing factor for eDNA in *S. aureus* is cell lysis, which depends on hydrolases under the control of *cidBAC* operon [68]. Similarly, the release of eDNA from *Escherichia faecalis* depends on metalloprotease, gelatinase (GelE), and serine protease (SprE) [69]. GelE positively regulates biofilm formation by activating autolysis, whereas SprE negatively regulates autolysis, eDNA release, and biofilm maturation [69]. Thomas et al. proposed two models for eDNA release: autolytic pathway and fratricidal pathway [69]. The autolytic pathway involves localizing GelE on the cell wall to activate autolysis by triggering a putative autolysin on the cell surface. GelE diffuses from the producer cell to a sibling cell in the fratricidal pathway, where the sibling cell undergoes autolysis by the GelE-mediated activation of autolysin [69]. Under *in vitro* conditions, *Acetobacter baumannii* produces membrane vesicles containing eDNA, increasing the total availability of eDNA [70]. eDNA plays a major role in conferring structural integrity to biofilms [63]. It facilitates aggregation and cell adhesion to various surfaces by providing thermodynamically favorable conditions [63,67]. Using confocal laser scanning electron microscopy, Allesen-Holm et al. demonstrated the localization of eDNA within *P. aeruginosa* biofilms [65]. When grown in flow chambers, *P. aeruginosa* biofilms developed mushroom-shaped structures where eDNA was primarily concentrated on the outer part of the mushroom's stalk, providing rigidity to the biofilm framework [65]. In *S. epidermidis,* eDNA is required for initial attachment and biofilm development [44]. Likewise, in *S. aureus*, β-toxins form covalent crosslinks in the presence of eDNA to produce an insoluble nucleoprotein matrix that enhances biofilm formation [71]. Similarly, in different strains *Clostridium difficile*, varied concentrations of eDNA were found to play a significant role in biofilm matrix integrity, with a positive correlation between eDNA, biofilm biomass, and sporulation frequency within the biofilm matrix [72].

Apart from structural contribution, eDNA is involved in several functions attributed to the bacterial community residing in the biofilm. Horizontal gene transfer among naturally competent cells is one of the main functions of eDNA that confers resistance to antibiotics [73]. The resistant genes are further propagated through mutants and eDNA released from the mutants, thereby expanding the resistance in the population [5]. eDNA also facilitates the chelation of $Mg^{2+}$ ions, which triggers gene expression for virulence factors and increased resistance to positively charged antibiotics and antimicrobial peptides [74]. eDNA plays a critical role in *P. aeruginosa* biofilms [75,76]. It mediates

cell-to-cell interaction and stabilization, binds to type IV pili and helps in motility, facilitates pyocyanin intercalation, and promotes aggregation [75,76]. eDNA interacts with curli amyloid fibrils in *S. Typhimurium* to form a stable eDNA-curli complex helps in stabilisation of biofilms [77].

The exogenous addition of DNase to growing or mature biofilm leads to either disruption of cells or biofilm inhibition [78]. The disruption of cells depends on the age of biofilm since the viable cells in young biofilms get easily detached due to DNase treatment compared to the cells in mature biofilms. Since eDNA plays an essential role in biofilm formation, the degradation of eDNA can be a potential strategy for developing novel therapeutics against biofilm-associated infections.

## 5. Methodologies to image and analyze biofilm matrix components

Understanding the composition and spatial distribution of biofilm components is essential to interpret the complexity of ECM and developing strategies for biofilm disruption. The biofilm micro-structures and constant change in composition and complexity make it a difficult model for imaging and analysis. However, the advent of advanced microscopy techniques has enabled us to characterize the physical and chemical organization of ECM. Combining spectroscopic and microscopic techniques results in an overall understanding of matrix components and their structural arrangement. Here, we describe a few microscopy and spectroscopy-based modalities commonly used to visualize the structural organization of ECM components and analyze biofilm formation. These modalities have expanded and deepened our understanding of complex phenomenon of biofilm biogenesis, colonization, arrangements of communities within biofilm, biofilm structural stabilities, and inter- or intra-species cell-to-cell interactions. The advantages and disadvantages of the modalities are summarized in Table 1.

### 5.1. Biofilm analysis based on spectroscopic methods

Spectroscopic techniques such as Fourier transform infrared (FTIR), Nuclear magnetic resonance (NMR), and Raman spectroscopy play a pivotal role in understanding the complex architecture and behavior of biofilms, since these techniques are non-invasive and non-destructive [79]. Based on IR, FTIR monitors time-dependent variation in EPS and provides a specific vibrational pattern contributed by functional groups of biomolecules in the matrix [80]. Wet biofilm samples are not preferred in FTIR as water absorbs strongly at mid-IR wavelength; however, attenuated total reflection-FTIR (ATR-FTIR) and microfluidic flow cells are now being used to overcome water interference to a certain extent [81]. Solid-state NMR has also been used to get insights into polysaccharide linkages and the structure of biofilms [82]. However, the low signal-to-noise ratio and time required for data acquisition are the major bottlenecks to using NMR for biofilm matrix analysis [83]. Raman Spectroscopy is used to identify the chemical composition of the biofilm [84]. It provides information based on molecular vibrations and rotations generated due to the Raman-scattering effect. The discovery of surface-enhanced Raman-scattering (SERS) nanosensors has significantly enhanced the limited sensitivity of Raman spectroscopy for biofilm analysis [85]. Pereira et al. found that the chemical composition of biofilms may be evaluated by observing the frequency of the scattered light, which differs for various molecules [86]. The blue-green redox-active chemical pyocyanin is responsible for the survival and pathogenicity of the bacterium strain *P. aeruginosa* PA14; in another study Bodelón et al. utilized surface-enhanced Raman scattering to identify this molecule [87]. Using *Geobacter sulfurreducens* as a model biofilm-forming bacterial strain, Robuschi et al. investigated confocal Raman spectroscopy's applicability to identify electrically active biofilm connection [88].

**Table 1**
Advantages and disadvantages of biofilm detection techniques [102–105].

| Techniques | Advantages | Disadvantages |
|---|---|---|
| Fourier transform infrared spectroscopy (FTIR-spectroscopy) | • Investigation of *in situ* biofilm development<br>• Non-invasive and Non-destructive<br>• Continuously monitored to give an accurate time frame of biofilm development | • Spectral overlap of vibrations of molecules in EPS and the cytoplasm<br>• 3D structure or thickness information is not available<br>• Tedious sample preparation |
| Nuclear magnetic resonance (NMR) | • Non-invasive and non-destructive<br>• Best Suited for *In situ* studies | • Low sensitivity and time-consuming<br>• Biofilm surface curvature can influence the results |
| Surface-enhanced Raman-scattering (SERS) | • High sensitivity and speed<br>• Minimal requirements for sample preparation<br>• Non-destructive<br>• Real-time detection without external labelling | • Expensive<br>• Special equipment is needed<br>• Focusses on a small area<br>• Time-consuming |
| Confocal laser scanning microscopy (CLSM) | • Useful for *in situ* and *in vivo* studies<br>• Hydrated biofilms can be imaged<br>• Resolution compatible with single-cell visualization<br>• Spatial distribution of 3-D images<br>• Automation *via* computer-enhanced digital image analysis | • Relatively slow<br>• Interference with auto-fluorescence<br>• The usage of fluorophores is needed<br>• The number of fluorescence filter combinations is limited<br>• Interference of local properties of the biofilm with the fluorescent probes<br>• Incompatible with opaque biofilms |
| Atomic force microscopy (AFM) | • Non-destructive technique<br>• Works under ambient conditions<br>• Provides 3-D reconstruction with high resolution<br>• Qualitative and quantitative evaluation of biofilm interaction | • Unable to acquire a large area scan<br>• Artifacts or sample damage caused by tip shape and size<br>• Expensive<br>• Samples become dehydrated during scanning |
| Scanning electron microscopy (SEM) | • Higher resolution<br>• Large depth of field<br>• Capability to visualize complex shapes<br>• Wide range of magnifications (20x to 30,000x)<br>• 3-D data measurement and quantification | • Time-consuming sample preparation<br>• Lacks vertical resolution<br>• The sample preparation process can destroy sample structure or cause artifacts<br>• Expensive and Destructive technique<br>• Unable to obtain scan over a larger area |
| Scanning transmission X-ray microscopy (STXM) | • Images of fully hydrated biological materials<br>• Provides spatial resolution of $< 50$ nm<br>• Allows chemical species to be mapped based on bonding structures<br>• Minimum sample preparation<br>• Does not need the addition of absorptive, reflective, or fluorescent probes | • No 3D-imaging<br>• Lower resolution than TEM<br>• Lacks vertical resolution<br>• The sample preparation process can destroy sample structure or cause artifacts<br>• Destructive technique |
| Environmental scanning electron microscopy (ESEM) | • Retains biofilm integrity<br>• No pre-treatment or sample preparation required<br>• Visualization of images of hydrated and non-conductive living bacterial biofilms at high | • Decreased resolution due to shortage of conductivity in wet samples<br>• Destructive techniques<br>• Specimen damage by focused electron beam at |

(*continued on next page*)

**Table 1** (*continued*)

| Techniques | Advantages | Disadvantages |
|---|---|---|
| | magnification and resolution | high magnification due to lack of metal coating |
| Cryo-scanning electron microscopy (Cryo-SEM) | • Simpler and quicker sample preparation than with conventional SEM, resulting in less sample destruction and artifacts | • Lower image resolution than traditional SEM<br>• Highly expensive<br>• The heat produced by the focused electron beam may melt the frozen surface of the sample |

### 5.2. Biofilm imaging using microscopy

The wide selection of fluorescence probes is used to study biofilms. Different probes with varied affinities can highlight biofilm composition, such as polysaccharides, nucleic acids, proteins, lipids, structural organization, cell viability, and specific enzymatic or metabolic activity. Fluorescent probes that include wheat germ agglutinin, calcofluor, dextran, and lectin can target polysaccharides in the extracellular polymeric substance to visualise the biofilm three-dimensional structure and provide details of like roughness, thickness, and biovolume. Syto-9 and 4′,6-diamidino-2-phenylindole are commonly used to stain biofilm microbial cells. Together with propidium iodide (PI), these dyes can determine the biofilm live/dead cell ratio and help in analyzing biofilms [89].

Laser scanning microscopy (LSM) has emerged as an essential tool to image and analyze three-dimensional architecture, matrix composition, and internal structures, including voids and channels of microbial communities within the biofilm [90]. Different variations in LSM, such as confocal laser scanning microscopy (CLSM), two-photon or multi-photon laser scanning microscopy (2PLSM), make it an essential technique for gaining in-depth knowledge on microbial biofilms [91]. Intrinsic properties of biofilms, such as reflection and autofluorescence, are advantageous for screening and imaging [91]. Traditional fluorescent probes and advanced probes-based cell permeability provide a detailed impression of the biofilm composition [92]. EPS components are screened using flour-labeled polymers, fluorescent beads, and EPS-specific glycoconjugates [91]. The capacity to spatially identify and detect distinct species or components (*e.g.*, genes) inside biofilms without damaging them has been a significant achievement in biofilm research [91]. In situ, fluorescence imaging can reveal biofilm structure and microorganism function. The fluorescence *in situ* hybridization (FISH) technique based on CLSM helps to visualize mixed-species biofilms. FISH allows the evaluation of competition and cooperation in the biofilm community [93]. CLSM has become a versatile tool for studying biofilm formation and is a preferred method to image biofilm since it offers flexible mounting and non-invasive sectioning of the samples. CLSM merges layer-by-layer scanning and sample imaging to reflect the spatial distribution of matrix components tagged with fluorescent probes [94]. The fluorescent probes are designed with minimal interference, high sensitivity, and specificity, providing highly resolved 3D mapping and quantifying various biomolecules in the matrix [94]. Amphiphilic carbon-based quantum dots in CSLM have shown exceptionally enhanced spatial resolution in *P. aeruginosa* biofilms [95]. When 3D images are taken across a specific period, CLSM provides a 3-D representation of the biofilm and depicts the real-time variation (4D imaging) [10]. However, with no prior information on matrix components, the specificity and choice of fluorophores become challenging. Recent research by Pan et al. (2022) used confocal laser scanning microscopy to identify biofilm architecture and extracellular polymeric material substances. One limitation of confocal laser scanning microscopy is the laser's penetration depth, even if it is feasible to examine biofilm samples at different depths. Visibility is reduced because samples thicker than 200 μm absorb the laser light quickly [96]. A comprehensive review of the utilization of laser scanning microscopy to delineate the structural elements of matrix components is described elsewhere [90].

High-end microscopy techniques engaged in imaging fluorescently labeled and unlabeled ECM components have the potential to analyze biofilm matrix with a spatiotemporal resolution [97]. EPS surrounding the cells can be observed using a Scanning Electron Microscope (SEM). Due to the high resolution and aid of image analysis tools, SEM images help assess the effects of anti-biofilm treatments [93]. SEM uses a beam of electrons to scan the surface and provides topographical information about the biofilm matrix [93]. It is the choice of tool to study bacterial adherence patterns and binding capacity when it is colonized to form biofilms on nanocomposite coatings and textured surfaces [93]. In recent research, SEM was used to observe biofilms, which exposed the crucial functions of bacterial cells and electrode pili in electron transport [98]. The considerable depth of field in SEM provides a better spatial resolution of matrix structure with the possibility to visualize individual cells within the biofilm [93]. However, it does not provide information about the chemical nature or functionality of the EPS components [93].

Additionally, SEM based-imaging techniques require biofilms to be dried and coated with a conductive material, altering biofilm architecture. The shortcomings in SEM can be overcome by the use of its variants, such as Environmental SEM (ESEM), Variable pressure-SEM (VP-SEM), and cryo-SEM [10]. In VP-SEM, the samples are stained and coated with metals without dehydration, preserving the 3D structure with minimal loss of structural information to conventional SEM [10]. ESEM does not require any pre-treatment of samples and, therefore, preserves the sample state to maintain the integrity of the biofilm matrix [95]. However, the image resolution and integrity are affected over imaging time because no metal coating results in conductivity. Therefore, the image needs to be captured quickly [99].

On the other hand, cryo-SEM is based on rapidly frozen samples under high pressure, resulting in the generation of descriptive images with the ultrastructure comprising cells and interactive matrix components [100]. The drawback of cryo-SEM is the possibility of melting the sample due to the enormous heat generated during imaging *via* the focused electron beam [10]. STXM, complemented with X-ray absorption near-edge structures technology (XANES), becomes a highly suitable analytical tool for examining hydrated biofilms [91]. XANES can provide detailed information on biofilm chemistry, such as chemical bonds, speciation, charge, and magnetic state of molecules [91,97]. STXM provides spatial and chemical resolution, which can be inferred as a compositional mapping of various biomolecules in the matrix [97]. The sample size and thickness of the biofilm matrix pose a real challenge for STXM [91].

Atomic force microscopy (AFM) has developed into a potent method that produces topographic pictures of 3D surface morphology. Force-distance curves are utilized to show how the substratum interacts with molecules during the formation of biofilms [101]. Therefore, AFM can provide qualitative and quantitative information on biofilm biomass grown on different substrates with different bacterial species [93]. It can estimate surface roughness and is a convenient technique to image biofilm samples; however, complementary tools are needed to get more in-depth information. The efficacy of AFM is drastically enhanced by coupling it with CSLM and other spectroscopic techniques [93]. Combining AFM and vibrational spectroscopic methods such as IR, Raman spectroscopy offers a detailed understanding of bacterial state and topographical and chemical features of biofilm [93].

### 5.3. Biofilm image analysis

The analysis tools provide a fantastic platform to extract additional information from the 3D images of biofilms. In recent years, there has been an exponential increase in the number of software that can analyze the biofilm matrix and provide valuable insights into the structure. However, each analysis tool has its benefits and weaknesses, as summarized in Table 2. Here, we briefly describe the tools and software used

**Table 2**
Advantages and disadvantages of biofilm image analysis software.

| Image Analysis Software | Advantages | Disadvantages | Reference |
|---|---|---|---|
| daime (digital image analysis in microbial ecology) | • Integrates image processing, image analysis, and 3D visualization<br>• Independent of shapes: can measure the distribution of single cells, filamentous or clumps of cells<br>• Spatial analysis of complex samples<br>• It can be used in identifying the biofilm of microorganisms involved in mutualistic interactions | • The image number and size to be analyzed are limited by the memory of the computer<br>• Some vertically stratified biofilms require modification in a spatial arrangement | Daims et al. 2006 |
| BioFilmAnalyzer | • Evaluation of cell count and live/dead ratio<br>• Allows quantification of fluorescent<br>• protein in the subpopulation of cells<br>• Fast and easy to handle | • Image pre-processing leads to increased background noise | Bogachev et al. 2018 |
| BAIT (Biofilm Architecture Inference Tool) | • Measures the architecture of the matrix<br>• It uses a new thresholding algorithm that overcomes the disadvantages of traditional methods | • The current version of BAIT can only analyze individual channels for biofilm analysis | Luo et al. 2019 |
| BiofilmQ | • Open-source software<br>• Focus on cytometry, analysis, and visualization of microbial community<br>• Can analyze both 2D and 3D images<br>• Toolset for both spatial and spatiotemporal analysis of microbial communities<br>• User-friendly: Researchers with less expertise in the field of programming can also handle | • The software requires identification of the biovolume of the biofilm either *via* a single fluorescence channel or importing the images after the segmentation process<br>• The result of image analysis dramatically depends upon the quality of the segmented images | Hartmann et al. 2021 |

to understand the complex structure and phenomenon of biofilm formation.

*daime (Digital image analysis in microbial ecology):* This tool works with 2D and 3D images of the biofilm matrix. It allows users to segregate objects from the images, which can be later segmented and used for further analysis, such as spatial arrangement pattern generation and abundance quantification. This analysis helps in real-time 3D-image generation after Z-stacking in CLSM [106].

*BioFilmAnalyzer:* This is a free software tool for counting fluorescently labeled cells from microphotographs. The values are very close to expert manual counting and flow cytometry. It is a valuable tool for getting a precise count of live and dead cells within the biofilm matrix. It could also be used for analyzing differential gene expression across the biofilm matrix over time using labeled reporter genes [107].

*BAIT (Biofilm Architecture Inference Tool):* This image analysis tool uses a well-defined MATLAB-based algorithm to measure bio-volume, the total number and surface area of objects, connectivity, and porosity in the matrix fluffiness, and viability from the CLSM images. This tool also enhances the signal-to-noise ratio by incorporating a specific algorithm that allows the creation of biofilm boundaries, thereby helping the software distinguish biofilm matrix components from the background [108].

*BiofilmQ:* This is a highly advanced quantitative tool for processing, analyzing, and visualizing CLSM images of biofilms. Given the bio-volume from CLSM images, it analyses spatial features of objects such as distance from each other, their location, density, roughness, and quantitative features like the abundance of the object locally and globally [109].

## 6. Conclusion

The biofilm matrix serves as an elastic scaffold that maintains the structure and stability of biofilm and provides a unique environment for bacteria to live a protected life. Extracellular polymeric substances (EPS) impart various functions crucial for biofilm assembly, persistence, and virulence. It allows the bacterial cells to reside in proximity, enabling adhesion, cohesion, cell-to-cell interaction, gene regulation, nutrient distribution, antibiotic resistance, and social interactions. With significant advancements in technology, it is now possible to get a deeper understanding of the architecture and composition of biofilms, which is an absolute need of the hour to open new gateways for drug discovery against chronic biofilm-associated diseases.

## Author contribution

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

## CRediT authorship contribution statement

**Harshita Agarwal:** Writing – review & editing, Writing – original draft, Visualization. **Bharat Gurnani:** Writing – review & editing, Writing – original draft. **Bhumika Pippal:** Writing – review & editing. **Neha Jain:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

*Research funding*

None declared.

*Acknowledgments*

We thank the Jain lab members for critically reading the manuscript.. We duly acknowledge senior research fellowship from Council of Scientific & Industrial Research (CSIR), Ministry of Science and Technology to HA, Prime minister Research Fellowship (PMRF), Ministry of Education to BP and junior research fellowship from Ministry of AYUSH (S/MOA/MTM/AA/20210105) to BG through CoE-AyurTech.

## Data availability

No data was used for the research described in the article.

## References


[1] M.R. Parsek, P.K. Singh, Bacterial biofilms: an emerging link to disease pathogenesis, Annu. Rev. Microbiol. 57 (1) (2003) 677–701, https://doi.org/10.1146/annurev.micro.57.030502.090720.

[2] L. Hall-Stoodley, J.W. Costerton, P. Stoodley, Bacterial biofilms: from the natural environment to infectious diseases, Nat. Rev. Microbiol. 2 (2) (2004) 95–108, https://doi.org/10.1038/nrmicro821.

[3] I. Sutherland, Biofilm exopolysaccharides: a strong and sticky framework, Microbiol. Read. Engl. 147 (Pt 1) (2001) 3–9, https://doi.org/10.1099/00221287-147-1-3.

[4] O.E. Petrova, K. Sauer, Sticky situations: key components that control bacterial surface attachment, J. Bacteriol. 194 (10) (2012) 2413–2425, https://doi.org/10.1128/JB.00003-12.

[5] C. Michaelis, E. Grohmann, Horizontal gene transfer of antibiotic resistance genes in biofilms, Antibiotics 12 (2) (2023) 328, https://doi.org/10.3390/antibiotics12020328.

[6] C.W. Hall, T.-F. Mah, Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria, FEMS Microbiol. Rev. 41 (3) (2017) 276–301, https://doi.org/10.1093/femsre/fux010.

[7] C. de la Fuente-Núñez, F. Reffuveille, L. Fernández, R.E. Hancock, Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies, Curr. Opin. Microbiol. 16 (5) (2013) 580–589, https://doi.org/10.1016/j.mib.2013.06.013.

[8] A.G. Goswami, S. Basu, T. Banerjee, V.K.Biofilm Shukla, Wound Healing, From bench to bedside, Eur. J. Med. Res. 28 (1) (2023) 157, https://doi.org/10.1186/s40001-023-01121-7.

[9] J. Lemos, S. Palmer, L. Zeng, Z. Wen, J. Kajfasz, I. Freires, J. Abranches, L. Brady, The biology of *Streptococcus mutans*, Microbiol. Spectr. 7 (1) (2019), https://doi.org/10.1128/microbiolspec.GPP3-0051-2018.

[10] M. Relucenti, G. Familiari, O. Donfrancesco, M. Taurino, X. Li, R. Chen, M. Artini, R. Papa, L. Selan, Microscopy methods for biofilm imaging: focus on SEM and VP-SEM pros and cons, Biology (Basel) 10 (1) (2021) 51, https://doi.org/10.3390/biology10010051.

[11] Y. Liu, S.-F. Yang, Y. Li, H. Xu, L. Qin, J.-H. Tay, The influence of cell and substratum surface hydrophobicities on microbial attachment, J. Biotechnol. 110 (3) (2004) 251–256, https://doi.org/10.1016/j.jbiotec.2004.02.012.

[12] R.S. Friedlander, N. Vogel, J. Aizenberg, Role of flagella in adhesion of *Escherichia coli* to abiotic surfaces, Langmuir 31 (22) (2015) 6137–6144, https://doi.org/10.1021/acs.langmuir.5b00815.

[13] R.M. Donlan, J.W. Costerton, Biofilms: survival mechanisms of clinically relevant microorganisms, Clin. Microbiol. Rev. 15 (2) (2002) 167–193, https://doi.org/10.1128/CMR.15.2.167-193.2002.

[14] G.R. Amores, A. de las Heras, A. Sanches-Medeiros, A. Elfick, R. Silva-Rocha, Systematic identification of novel regulatory interactions controlling biofilm formation in the bacterium *Escherichia coli*, Sci. Rep. 7 (1) (2017) 16768, https://doi.org/10.1038/s41598-017-17114-6.

[15] N. Rabin, Y. Zheng, C. Opoku-Temeng, Y. Du, E. Bonsu, H.O. Sintim, Biofilm formation mechanisms and targets for developing antibiofilm agents, Future Med. Chem. 7 (4) (2015) 493–512, https://doi.org/10.4155/fmc.15.6.

[16] M. Kostakioti, M. Hadjifrangiskou, S.J. Hultgren, Bacterial biofilms: development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era, Cold Spring Harb. Perspect. Med. 3 (4) (2013) a010306, https://doi.org/10.1101/cshperspect.a010306.

[17] D. McDougald, S.A. Rice, N. Barraud, P.D. Steinberg, S. Kjelleberg, Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal, Nat. Rev. Microbiol. 10 (1) (2011) 39–50, https://doi.org/10.1038/nrmicro2695.

[18] B.K. Hammer, B.L. Bassler, Quorum sensing controls biofilm formation in *Vibrio cholerae*, Mol. Microbiol. 50 (1) (2003) 101–104, https://doi.org/10.1046/j.1365-2958.2003.03688.x.

[19] H.C.;. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S.A. Rice, S. Kjelleberg, Biofilms: an emergent form of bacterial life, Nat. Rev. Microbiol. 14 (9) (2016) 563–575, https://doi.org/10.1038/nrmicro.2016.94.

[20] R. Nassar, M. Hachim, M. Nassar, E.G. Kaklamanos, M. Jamal, D. Williams, A. Senok, Microbial metabolic genes crucial for *S. aureus* biofilms: an insight from *Re*-analysis of publicly available microarray datasets, Front. Microbiol. 11 (2021), https://doi.org/10.3389/fmicb.2020.607002.

[21] M.R. Parsek, E.P. Greenberg, Sociomicrobiology: the connections between quorum sensing and biofilms, Trends Microbiol 13 (1) (2005) 27–33, https://doi.org/10.1016/j.tim.2004.11.007.

[22] U. Romling, M.Y. Galperin, M. Gomelsky, Cyclic Di-GMP: the first 25 years of a universal bacterial second messenger, Microbiol. Mol. Biol. Rev. 77 (1) (2013) 1–52, https://doi.org/10.1128/mmbr.00043-12.

[23] J.R. Chambers, K. Sauer, Small RNAs and their role in biofilm formation, Trends Microbiol (2013) 39–49, https://doi.org/10.1016/j.tim.2012.10.008.

[24] W.-L. Ng, B.L. Bassler, Bacterial quorum-sensing network architectures, Annu. Rev. Genet. 43 (1) (2009) 197–222, https://doi.org/10.1146/annurev-genet-102108-134304.

[25] K. Vadakkan, A.K. Ngangbam, K. Sathishkumar, N.P. Rumjit, M.K. Cheruvathur, A review of chemical signaling pathways in the quorum sensing circuit of *Pseudomonas aeruginosa*, Int. J. Biol. Macromol. 254 (Pt 2) (2024) 127861, https://doi.org/10.1016/j.ijbiomac.2023.127861.

[26] G. İnat, B. Sırıken, C. Başkan, İ. Erol, T. Yıldırım, A. Çiftci, Quorum sensing systems and related virulence factors in *Pseudomonas aeruginosa* isolated from chicken meat and ground beef, Sci. Rep. 11 (1) (2021) 15639, https://doi.org/10.1038/s41598-021-94906-x.

[27] C.H. Hoe, C.A. Raabe, T.S. Rozhdestvensky, T.H. Tang, Bacterial sRNAs: regulation in stress, Int. J. Med. Microbiol. (2013) 217–229, https://doi.org/10.1016/j.ijmm.2013.04.002.

[28] M.A. Ghaz-Jahanian, F. Khodaparastan, A. Berenjian, H. Jafarizadeh-Malmiri, Influence of small RNAs on biofilm formation process in bacteria, Mol. Biotech. (2013) 288–297, https://doi.org/10.1007/s12033-013-9700-6.

[29] D.H. Lenz, K.C. Mok, B.N. Lilley, R.V. Kulkarni, N.S. Wingreen, B.L. Bassler, The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*, Cell 118 (1) (2004) 69–82, https://doi.org/10.1016/j.cell.2004.06.009.

[30] B.R. Borlee, A.D. Goldman, K. Murakami, R. Samudrala, D.J. Wozniak, M. R. Parsek, *Pseudomonas aeruginosa* Uses a Cyclic-Di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix, Mol. Microbiol. 75 (4) (2010) 827–842, https://doi.org/10.1111/j.1365-2958.2009.06991.x.

[31] M. Valentini, A. Filloux, Biofilms and cyclic Di-GMP (c-Di-GMP) signaling: lessons from *Pseudomonas aeruginosa* and other bacteria, J. Biol. Chem. (2016) 12547–12555, https://doi.org/10.1074/jbc.R115.711507.

[32] R. Hengge, Principles of C-Di-GMP signalling in bacteria, Nat. Rev. Microbiol. (2009) 263–273, https://doi.org/10.1038/nrmicro2109.

[33] M. Potts, Desiccation tolerance of prokaryotes, Microbiol. Rev. 58 (4) (1994) 755–805.

[34] E. O'Neill, C. Pozzi, P. Houston, H. Humphreys, D.A. Robinson, A. Loughman, T. J. Foster, J.P. O'Gara, A Novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB, J. Bacteriol. 190 (11) (2008) 3835–3850, https://doi.org/10.1128/JB.00167-08.

[35] T.V. Sønderby, Z. Najarzadeh, D.E. Otzen, Functional bacterial amyloids: understanding fibrillation, regulating biofilm fibril formation and organizing surface assemblies, Molecules 27 (13) (2022) 4080, https://doi.org/10.3390/molecules27134080.

[36] Ü. Akbey, M. Andreasen, Functional amyloids from bacterial biofilms – structural properties and interaction partners, Chem. Sci. 13 (22) (2022) 6457–6477, https://doi.org/10.1039/D2SC00645F.

[37] S. Bhoite, N. Van Gerven, M.R. Chapman, H. Remaut, Curli biogenesis: bacterial amyloid assembly by the type VIII secretion pathway, Eco. Sal. Plus 8 (2) (2019), https://doi.org/10.1128/ecosalplus. ESP-0037–2018.

[38] C.B. Rasmussen, G. Christiansen, B.S. Vad, C. Lynggaard, J.J. Enghild, M. Andreasen, D. Otzen, Imperfect repeats in the functional amyloid protein FapC reduce the tendency to fragment during fibrillation, Protein Sci 28 (3) (2019) 633–642, https://doi.org/10.1002/pro.3566.

[39] G. Zeng, B.S. Vad, M.S. Dueholm, G. Christiansen, M. Nilsson, T. Tolker-Nielsen, P.H. Nielsen, R.L. Meyer, D.E. Otzen, Functional bacterial amyloid increases pseudomonas biofilm hydrophobicity and stiffness, Front. Microbiol. (2015) 6.

[40] C.B. Rasmussen, G. Christiansen, B.S. Vad, C. Lynggaard, J.J. Enghild, M. Andreasen, D. Otzen, Imperfect repeats in the functional amyloid protein FapC reduce the tendency to fragment during fibrillation, Protein Sci 28 (3) (2019) 633–642, https://doi.org/10.1002/pro.3566.

[41] J. Cámara-Almirón, Y. Navarro, L. Díaz-Martínez, M.C. Magno-Pérez-Bryan, C. Molina-Santiago, J.R. Pearson, A. de Vicente, A. Pérez-García, D. Romero, Dual functionality of the amyloid protein TasA in bacillus physiology and fitness on the phylloplane, Nat. Commun. 11 (1) (2020) 1859, https://doi.org/10.1038/s41467-020-15758-z.

[42] N. Salinas, J.-P. Colletier, A. Moshe, M. Landau, Extreme amyloid polymorphism in *Staphylococcus aureus* virulent PSMα peptides, Nat. Commun. 9 (1) (2018) 3512, https://doi.org/10.1038/s41467-018-05490-0.

[43] E. Arad, K.B. Pedersen, O. Malka, S. Mambram Kunnath, N. Golan, P. Aibinder, B. Schiøtt, H. Rapaport, M. Landau, R. Jelinek, *Staphylococcus aureus* functional amyloids catalyze degradation of β-lactam antibiotics, Nat. Commun. 14 (1) (2023) 8198, https://doi.org/10.1038/s41467-023-43624-1.

[44] Z. Qin, Y. Ou, L. Yang, Y. Zhu, T. Tolker-Nielsen, S. Molin, D. Qu, Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*, Microbiol. Read. Engl. 153 (Pt 7) (2007) 2083–2092, https://doi.org/10.1099/mic.0.2007/006031-0.

[45] A.R. Markande, V.R. Vemuluri, Y.S. Shouche, A.S. Nerurkar, Characterization of *solibacillus silvestris* strain AM1 that produces amyloid bioemulsifier, J. Basic Microbiol. 58 (6) (2018) 523–531, https://doi.org/10.1002/jobm.201700685.

[46] C.R. Schaeffer, K.M. Woods, G.M. Longo, M.R. Kiedrowski, A.E. Paharik, H. Büttner, M. Christner, R.J. Boissy, A.R. Horswill, H. Rohde, P.D. Fey, Accumulation-associated protein enhances *Staphylococcus epidermidis* biofilm formation under dynamic conditions and is required for infection in a rat catheter model, Infect. Immun. 83 (1) (2014) 214–226, https://doi.org/10.1128/iai.02177-14.

[47] D.J. Little, G. Li, C. Ing, B.R. DiFrancesco, N.C. Bamford, H. Robinson, M. Nitz, R. Pomès, P.L. Howell, Modification and periplasmic translocation of the biofilm exopolysaccharide poly-β-1,6-N-Acetyl-D-glucosamine, Proc. Natl. Acad. Sci. U. S. A. 111 (30) (2014) 11013–11018, https://doi.org/10.1073/pnas.1406388111.

[48] K.M. Colvin, Y. Irie, C.S. Tart, R. Urbano, J.C. Whitney, C. Ryder, P.L. Howell, D. J. Wozniak, M.R. Parsek, The pel and psl polysaccharides provide *Pseudomonas aeruginosa* structural redundancy within the biofilm matrix, Environ. Microbiol. 14 (8) (2012), https://doi.org/10.1111/j.1462-2920.2011.02657.x.

[49] O. Orgad, Y. Oren, S.L. Walker, M. Herzberg, The role of alginate in *Pseudomonas aeruginosa* EPS adherence, viscoelastic properties and cell attachment, Biofouling 27 (7) (2011) 787–798, https://doi.org/10.1080/08927014.2011.603145.

[50] C. Beloin, A. Roux, J.M. Ghigo, *Escherichia coli* Biofilms, Curr. Top. Microbiol. Immunol. 322 (2008) 249–289, https://doi.org/10.1007/978-3-540-75418-3_12.

[51] J.C.N. Fong, K.A. Syed, K.E. Klose, F.H. Yildiz, Role of Vibrio polysaccharide (Vps) Genes in VPS production, biofilm formation and *Vibrio cholerae* pathogenesis, Microbiology 156 (Pt 9) (2010) 2757–2769, https://doi.org/10.1099/mic.0.040196-0.

[52] M.B. Poulin, L.L. Kuperman, Regulation of biofilm exopolysaccharide production by cyclic di-guanosine monophosphate, Front. Microbiol. 12 (2021), https://doi.org/10.3389/fmicb.2021.730980.

[53] M.P. Zaborowski, L. Balaj, X.O. Breakefield, C.P. Lai, Extracellular vesicles: composition, biological relevance, and methods of study, BioScience 65 (8) (2015) 783–797, https://doi.org/10.1093/biosci/biv084.

[54] S.R. Schooling, T.J. Beveridge, Membrane vesicles: an overlooked component of the matrices of biofilms, J. Bacteriol. 188 (16) (2006) 5945–5957, https://doi.org/10.1128/JB.00257-06.

[55] Y. Tashiro, H. Uchiyama, N. Nomura, Multifunctional membrane vesicles in *Pseudomonas aeruginosa*, Environ. Microbiol. 14 (6) (2012) 1349–1362, https://doi.org/10.1111/j.1462-2920.2011.02632.x.

[56] N. Chen, Y. Li, X. Liang, K. Qin, Y. Zhang, J. Wang, Q. Wu, T.B. Gupta, Y. Ding, Bacterial extracellular vesicle: a non-negligible component in biofilm life cycle and challenges in biofilm treatments, Biofilm 8 (2024) 100216, https://doi.org/10.1016/j.bioflm.2024.100216.

[57] G. Kumaraguru, S. Moorthy, A.K. Krishna Kumar, J Malaiyandi, Chapter 14 - lipidomics profiling of microbial biofilm, in: H. Sarma, S. Joshi, D. Lahiri, R. R. Ray, M. Davoodbasha (Eds.), Microbial Biofilms, Academic Press, 2023, pp. 225–233, https://doi.org/10.1016/B978-0-323-95715-1.00007-8. EdsAdvances in Biotechnology and Bioengineering.

[58] A.A. Lattif, P.K. Mukherjee, J. Chandra, M.R. Roth, R. Welti, M. Rouabhia, M. A. Ghannoum, Lipidomics of candida albicans biofilms reveals phase-dependent production of phospholipid molecular classes and role for lipid rafts in biofilm formation, Microbiology 157 (11) (2011) 3232–3242, https://doi.org/10.1099/mic.0.051086-0.

[59] F. Dubois-Brissonnet, E. Trotier, R. Briandet, The Biofilm lifestyle involves an increase in bacterial membrane saturated fatty acids, Front. Microbiol. 7 (2016), https://doi.org/10.3389/fmicb.2016.01673.

[60] T. Matsuyama, Y. Nakagawa, Surface-active exolipids: analysis of absolute chemical structures and biological functions, J. Microbiol. Methods 25 (2) (1996) 165–175, https://doi.org/10.1016/0167-7012(95)00109-3.

[61] D.G. Davies, C.N.H. Marques, A fatty acid messenger is responsible for inducing dispersion in microbial biofilms, J. Bacteriol 191 (5) (2009) 1393–1403, https://doi.org/10.1128/jb.01214-08.

[62] L. Montanaro, P. Speziale, D. Campoccia, S. Ravaioli, I. Cangini, G. Pietrocola, S. Giannini, C.R. Arciola, Scenery of staphylococcus implant infections in orthopedics, Future Microbiol 6 (11) (2011) 1329–1349, https://doi.org/10.2217/fmb.11.117.

[63] A.L. Ibáñez de Aldecoa, O. Zafra, J.E. González-Pastor, Mechanisms and regulation of extracellular DNA release and its biological roles in microbial communities, Front. Microbiol. 8 (2017) 1390, https://doi.org/10.3389/fmicb.2017.01390.

[64] A. Devaraj, J.R. Buzzo, L. Mashburn-Warren, E.S. Gloag, L.A. Novotny, P. Stoodley, L.O. Bakaletz, S.D. Goodman, The extracellular DNA lattice of bacterial biofilms is structurally related to holliday junction recombination intermediates, Proc. Natl. Acad. Sci. U. S. A 116 (50) (2019) 25068–25077, https://doi.org/10.1073/pnas.1909017116.

[65] M. Allesen-Holm, K.B. Barken, L. Yang, M. Klausen, J.S. Webb, S. Kjelleberg, S. Molin, M. Givskov, T. Tolker-Nielsen, A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms, Mol. Microbiol. 59 (4) (2006) 1114–1128, https://doi.org/10.1111/j.1365-2958.2005.05008.x.

[66] K. Yang, L. Wang, X. Cao, Z. Gu, G. Zhao, M. Ran, Y. Yan, J. Yan, L. Xu, C. Gao, M. Yang, The origin, function, distribution, quantification, and research advances of extracellular DNA, Int. J. Mol. Sci. 23 (22) (2022) 13690, https://doi.org/10.3390/ijms232213690.

[67] T. Das, S. Sehar, M. Manefield, The roles of extracellular DNA in the structural integrity of extracellular polymeric substance and bacterial biofilm development, Environ. Microbiol. Rep. 5 (6) (2013) 778–786, https://doi.org/10.1111/1758-2229.12085.

[68] K.C. Rice, E.E. Mann, J.L. Endres, E.C. Weiss, J.E. Cassat, M.S. Smeltzer, K. W. Bayles, The cidA murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*, Proc. Natl. Acad. Sci. U. S. A. 104 (19) (2007) 8113–8118, https://doi.org/10.1073/pnas.0610226104.

[69] V.C. Thomas, L.R. Thurlow, D. Boyle, L.E. Hancock, Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development, J. Bacteriol. 190 (16) (2008) 5690–5698, https://doi.org/10.1128/JB.00314-08.

[70] P.K. Sahu, P.S. Iyer, A.M. Oak, K.R. Pardesi, B.A. Chopade, Characterization of eDNA from the clinical strain *Acinetobacter baumannii* AIIMS 7 and its role in biofilm formation, Sci. World J. 2012 (2012) 973436, https://doi.org/10.1100/2012/973436.

[71] M.J. Huseby, A.C. Kruse, J. Digre, P.L. Kohler, J.A. Vocke, E.E. Mann, K. W. Bayles, G.A. Bohach, P.M. Schlievert, D.H. Ohlendorf, C.A. Earhart, Beta toxin catalyzes formation of nucleoprotein matrix in staphylococcal biofilms, Proc. Natl. Acad. Sci. U. S. A. 107 (32) (2010) 14407–14412, https://doi.org/10.1073/pnas.0911032107.

[72] L.F. Dawson, J. Peltier, C.L. Hall, M.A. Harrison, M. Derakhshan, H.A. Shaw, N. F. Fairweather, B.W. Wren, Extracellular DNA, cell surface proteins and c-Di-GMP promote biofilm formation in *Clostridioides difficile*, Sci. Rep. 11 (1) (2021) 3244, https://doi.org/10.1038/s41598-020-78437-5.

[73] D.K. Sharma, Y.S. Rajpurohit, Multitasking functions of bacterial extracellular DNA in biofilms, J. Bacteriol. 206 (4) (2024) e00006, https://doi.org/10.1128/jb.00006-24. -24.

[74] M. Okshevsky, R.L. Meyer, The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms, Crit. Rev. Microbiol. 41 (3) (2015) 341–352, https://doi.org/10.3109/1040841X.2013.841639.

[75] K.B. Barken, S.J. Pamp, L. Yang, M. Gjermansen, J.J. Bertrand, M. Klausen, M. Givskov, C.B. Whitchurch, J.N. Engel, T. Tolker-Nielsen, Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms, Environ. Microbiol. 10 (9) (2008) 2331–2343, https://doi.org/10.1111/j.1462-2920.2008.01658.x.

[76] T. Das, M. Manefield, Pyocyanin promotes extracellular DNA release in *Pseudomonas aeruginosa*, PloS One 7 (10) (2012) e46718, https://doi.org/10.1371/journal.pone.0046718.

[77] S.A. Tursi, Ç. Tükel, Curli-containing enteric biofilms inside and out: matrix composition, immune recognition, and disease implications, Microbiol. Mol. Biol. Rev. MMBR 82 (4) (2018) e00028, https://doi.org/10.1128/MMBR.00028-18. -18.

[78] L. Hall-Stoodley, L. Nistico, K. Sambanthamoorthy, B. Dice, D. Nguyen, W. J. Mershon, C. Johnson, F. Ze Hu, P. Stoodley, G.D. Ehrlich, J.C. Post, Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in *Streptococcus pneumoniae* clinical isolates, BMC Microbiol 8 (2008) 173, https://doi.org/10.1186/1471-2180-8-173.

[79] P. Seredin, D. Goloshchapov, V. Kashkarov, D. Nesterov, Y. Ippolitov, I. Ippolitov, J. Vongsvivut, Effect of exo/endogenous prophylaxis dentifrice/drug and cariogenic conditions of patient on molecular property of dental biofilm: synchrotron FTIR spectroscopic study, Pharmaceutics 14 (7) (2022) 1355, https://doi.org/10.3390/pharmaceutics14071355.

[80] K. Loutherback, L. Chen, H.-Y.N. Holman, Open-channel microfluidic membrane device for long-term FT-IR spectromicroscopy of live adherent cells, Anal. Chem. 87 (9) (2015) 4601–4606, https://doi.org/10.1021/acs.analchem.5b00524.

[81] A. Dazzi, C.B. Prater, AFM-IR: technology and applications in nanoscale infrared spectroscopy and chemical imaging, Chem. Rev. 117 (7) (2017) 5146–5173, https://doi.org/10.1021/acs.chemrev.6b00448.

[82] C. Reichhardt, L. Cegelski, Solid-state NMR for bacterial biofilms, Mol. Phys. 112 (7) (2014) 887–894, https://doi.org/10.1080/00268976.2013.837983.

[83] K. Cm, H. Mp, H. R, B. At, G. E, W. Do, C. Sl, Situ detection of subsurface biofilm using low-field NMR: a field study, Environ. Sci. Technol. 49 (18) (2015), https://doi.org/10.1021/acs.est.5b02690.

[84] P. Desmond, J.P. Best, E. Morgenroth, N. Derlon, Linking composition of extracellular polymeric substances (EPS) to the physical structure and hydraulic resistance of membrane biofilms, Water Res (2018) 211–221, https://doi.org/10.1016/j.watres.2017.12.058.

[85] H. Pu, Y. Xu, D.-W. Sun, Q. Wei, X. Li, Optical nanosensors for biofilm detection in the food industry: principles, applications and challenges, Crit. Rev. Food Sci. Nutr. 61 (13) (2021) 2107–2124, https://doi.org/10.1080/10408398.2020.1808877.

[86] J. Pereira, S. de Nooy, T. Sleutels, A. ter Heijne, Opportunities for visual techniques to determine characteristics and limitations of electro-active biofilms, Biotechnol. Adv. 60 (2022) 108011, https://doi.org/10.1016/j.biotechadv.2022.108011.

[87] G. Bodelón, V. Montes-García, V. López-Puente, E.H. Hill, C. Hamon, M.N. Sanz-Ortiz, S. Rodal-Cedeira, C. Costas, S. Celiksoy, I. Pérez-Juste, L. Scarabelli, A. La Porta, J. Pérez-Juste, I. Pastoriza-Santos, L.M. Liz-Marzán, Detection and imaging of quorum sensing in *Pseudomonas aeruginosa* biofilm communities by surface-enhanced resonance raman scattering, Nat. Mater. 15 (11) (2016) 1203–1211, https://doi.org/10.1038/nmat4720.

[88] L. Robuschi, J.P. Tomba, J.P. Busalmen, Proving *Geobacter* biofilm connectivity with confocal raman microscopy, J. Electroanal. Chem. 793 (2017) 99–103, https://doi.org/10.1016/j.jelechem.2016.11.005.

[89] M. Chamlagain, J. Hu, R.V. Sionov, D. Steinberg, Anti-bacterial and anti-biofilm activities of arachidonic acid against the cariogenic bacterium *Streptococcus mutans*, Front. Microbiol. 15 (2024), https://doi.org/10.3389/fmicb.2024.1333274.

[90] T.R. Neu, J.R. Lawrence, Innovative techniques, sensors, and approaches for imaging biofilms at different scales, Trends Microbiol 23 (4) (2015) 233–242, https://doi.org/10.1016/j.tim.2014.12.010.

[91] T.R. Neu, B. Manz, F. Volke, J.J. Dynes, A.P. Hitchcock, J.R. Lawrence, Advanced imaging techniques for assessment of structure, composition and function in biofilm systems, FEMS Microbiol. Ecol. 72 (1) (2010) 1–21, https://doi.org/10.1111/j.1574-6941.2010.00837.x.

[92] H. Straub, F. Zuber, L. Eberl, K. Maniura-Weber, Q.In Ren, Situ investigation of *Pseudomonas aeruginosa* biofilm development: interplay between flow, growth medium, and mechanical properties of substrate, ACS Appl. Mater. Interfaces 15 (2) (2023) 2781–2791, https://doi.org/10.1021/acsami.2c20693.

[93] Y. Huang, S. Chakraborty, H. Liang, Methods to probe the formation of biofilms: applications in foods and related surfaces, Anal. Methods 12 (4) (2020) 416–432, https://doi.org/10.1039/C9AY02214G.

[94] M.-Y. Chen, D.-J. Lee, Z. Yang, X.F. Peng, J.Y. Lai, Fluorecent staining for study of extracellular polymeric substances in membrane biofouling layers, Environ. Sci. Technol. 40 (21) (2006) 6642–6646, https://doi.org/10.1021/es0612955.

[95] M. Ritenberg, S. Nandi, S. Kolusheva, R. Dandela, M.M. Meijler, R. Jelinek, Imaging *Pseudomonas aeruginosa* biofilm extracellular polymer scaffolds with amphiphilic carbon dots, ACS Chem. Biol. 11 (5) (2016) 1265–1270, https://doi.org/10.1021/acschembio.5b01000.

[96] M. Pan, H. Li, X. Han, W. Ma, X. Li, Q. Guo, B. Yang, C. Ding, Y. Ma, Effects of hydrodynamic conditions on the composition, spatiotemporal distribution of different extracellular polymeric substances and the architecture of biofilms, Chemosphere 307 (2022) 135965, https://doi.org/10.1016/j.chemosphere.2022.135965.

[97] P. Zhang, Y.-P. Chen, J.-H. Qiu, Y.-Z. Dai, B. Feng, Imaging the microprocesses in biofilm matrices, Trends Biotechnol 37 (2) (2019) 214–226, https://doi.org/10.1016/j.tibtech.2018.07.006.

[98] J. Wang, X. Yang, M. Cui, Y. Liu, X. Li, L. Zhang, G. Zhan, A high-sensitive and durable electrochemical sensor based on *Geobacter*-dominated biofilms for heavy metal toxicity detection, Biosens. Bioelectron. 206 (2022) 114146, https://doi.org/10.1016/j.bios.2022.114146.

[99] J. Pellé, M. Longo, N. Le Poul, C. Hellio, S. Rioual, B. Lescop, Electrochemical monitoring of the *Pseudomonas aeruginosa* growth and the formation of a biofilm in TSB media, Bioelectrochemistry 150 (2023) 108344, https://doi.org/10.1016/j.bioelechem.2022.108344.

[100] K. Hrubanova, J. Nebesarova, F. Ruzicka, V. Krzyzanek, The innovation of cryo-SEM freeze-fracturing methodology demonstrated on high pressure frozen biofilm, Micron Oxf. Engl. 1993 110 (2018) 28–35, https://doi.org/10.1016/j.micron.2018.04.006.

[101] S.A. James, L.C. Powell, C.J. Wright, S.A. James, L.C. Powell, C.J. Wright, Atomic Force Microscopy of Biofilms—Imaging, Interactions, and Mechanics, IntechOpen, 2016, https://doi.org/10.5772/63312.

[102] G. Wolf, J.G. Crespo, M.A.M Reis, Optical and spectroscopic methods for biofilm examination and monitoring, Re-Views Environ. Sci. Biotechnol. 1 (3) (2002) 227–251, https://doi.org/10.1023/A:1021238630092.

[103] M. Pan, L. Zhu, L. Chen, Y. Qiu, J. Wang, Detection techniques for extracellular polymeric substances in biofilms: a review, BioResources 11 (2016), https://doi.org/10.15376/biores.11.3.8092-8115.

[104] J. Azeredo, N.F. Azevedo, R. Briandet, N. Cerca, T. Coenye, A.R. Costa, M. Desvaux, G. Di Bonaventura, M. Hébraud, Z. Jaglic, M. Kačániová, S. Knøchel, A. Lourenço, F. Mergulhão, R.L. Meyer, G. Nychas, M. Simões, O. Tresse, C. Sternberg, Critical review on biofilm methods, Crit. Rev. Microbiol. 43 (3) (2017) 313–351, https://doi.org/10.1080/1040841X.2016.1208146.

[105] S. Achinas, S.K. Yska, N. Charalampogiannis, J. Krooneman, G.J.W. Euverink, A technological understanding of biofilm detection techniques: a review, Materials (Basel) 13 (14) (2020) 3147, https://doi.org/10.3390/ma13143147.

[106] H. Daims, S. Lücker, M. Wagner, a Daime, Novel image analysis program for microbial ecology and biofilm research, Environ. Microbiol. 8 (2) (2006) 200–213, https://doi.org/10.1111/j.1462-2920.2005.00880.x.

[107] M.I. Bogachev, V.Y. Volkov, O.A. Markelov, E.Y. Trizna, D.R. Baydamshina, V. Melnikov, R.R. Murtazina, P.V. Zelenikhin, I.S. Sharafutdinov, A.R. Kayumov, Fast and simple tool for the quantification of biofilm-embedded cells sub-populations from fluorescent microscopic images, PloS One 13 (5) (2018) e0193267, https://doi.org/10.1371/journal.pone.0193267.

[108] T.L. Luo, M. Hayashi, M. Zsiska, B. Circello, M. Eisenberg, C. Gonzalez-Cabezas, B. Foxman, C.F. Marrs, A.H. Rickard, Introducing BAIT (Biofilm Architecture Inference Tool): a software program to evaluate the architecture of oral multi-species biofilms, Microbiol. Read. Engl. 165 (5) (2019) 527–537, https://doi.org/10.1099/mic.0.000761.

[109] R. Hartmann, H. Jeckel, E. Jelli, P.K. Singh, S. Vaidya, M. Bayer, D.K.H. Rode, L. Vidakovic, F. Díaz-Pascual, J.C.N. Fong, A. Dragoš, O. Lamprecht, J. G. Thöming, N. Netter, S. Häussler, C.D. Nadell, V. Sourjik, Á.T. Kovács, F. H. Yildiz, K. Drescher, Quantitative image analysis of microbial communities with BiofilmQ, Nat. Microbiol. 6 (2) (2021) 151–156, https://doi.org/10.1038/s41564-020-00817-4.

[110] M. Guilbaud, P. Piveteau, M. Desvaux, S. Brisse, R. Briandet, Exploring the diversity of listeria monocytogenes biofilm architecture by high-throughput confocal laser scanning microscopy and the predominance of the honeycomb-like morphotype, Appl. Environ. Microbiol. 81 (5) (2015) 1813–1819, https://doi.org/10.1128/AEM.03173-14.

[111] P.M. den Reijer, M. Sandker, S.V. Snijders, M. Tavakol, A.P.A. Hendrickx, W.J. B. van Wamel, Combining in vitro protein detection and in vivo antibody detection identifies potential vaccine targets against *Staphylococcus aureus* during osteomyelitis, Med. Microbiol. Immunol. (Berl.) 206 (1) (2017) 11–22, https://doi.org/10.1007/s00430-016-0476-8.

[112] L. Galezowski, N. Recham, D. Larcher, J. Miot, F. Skouri-Panet, F. Guyot, Microbially induced mineralization of layered mn oxides electroactive in li batteries, Front. Microbiol. 11 (2020), https://doi.org/10.3389/fmicb.2020.02031.

[113] H. Fang, M. Fazeli, W. Cheng, L. Huang, H. Hu, Biostabilization and transport of cohesive sediment deposits in the three gorges reservoir, PLOS ONE 10 (11) (2015) e0142673, https://doi.org/10.1371/journal.pone.0142673.