Multi-omics tools for studying microbial biofilms: current perspectives and future directions

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Introduction

Microorganisms exist in two predominant lifestyles in nature, namely, the free-floating planktonic state and the surface-attached biofilm state (1). Until the 1970s, the predominant notion among microbiologists was that bacteria exist mostly in the free-floating planktonic state; however, the pioneering work of William Costerton established the concept of "biofilms" and has subsequently driven the notion that this is their preferred growth mode in nature (2). Biofilms are defined as microbial communities encased in extracellular matrix, and are formed at solid, liquid, and air interfaces. They can exist on biotic and abiotic surfaces, as well as microaggregates (3). The biofilm state confers numerous survival advantages to microorganisms under stress conditions, during nutrient deprivation, and antibiotic treatment. Physiology, growth, and behaviour of microbial biofilms differ vastly from their planktonic counterparts (4). Due to these advantages, biofilms are able to tolerate and persist in environments in which planktonic cells would ordinarily not.

The occurrence of a sessile growth mode in pathogenic microorganisms often complicates treatment strategies. Traditionally, acute infections involving planktonic microbes have been treated effectively using antibiotics. However, the growth of pathogenic microbes in biofilm state renders such treatments ineffective, leading to chronic infections (5). At least 65-80% of infectious diseases are associated with the biofilm state as per a Centers for Disease Control and Prevention (CDC) report (6). Biofilm-related infections cover a wide array of diseases including, but not limited to, catheter- and implant-associated infections, chronic wounds, chronic otitis media, chronic osteomyelitis, chronic rhinosinositis, recurrent urinary tract infection, endocarditis, and lung infections associated with cystic fibrosis. Together these infections affect millions of people worldwide, resulting in an unnecessarily high mobidity rate and increasingly large mortality rate (7).

Biofilm characteristics conferring increased drug resistance properties

The physical, chemical, and environmental stresses faced by microorganisms lead to phenotypic switch from planktonic to biofilm state, which helps the microbes to evade host defenses and exhibit antibiotic tolerance via certain hallmark biofilm characteristics. A typical biofilm consists of extracellular matrix (ECM), which confers protection against desiccation, antibiotics, biocides, ultraviolet radiation, and host immune responses through mechanical

stability, all of which contributes to recalcitrance (8,9). Another important biofilm characteristic associated with drug resistance is the presence of a dormant subpopulation called persisters. Persister cells were first discovered in *Staphylococcus* sp. (10,11) and more recently in *Escherichia coli*, *Pseudomonas aeruginosa*, *Lactobacillus acidophilus*, and *Candida* species (12–16). Since antibiotics are usually effective against metabolically active cells, the metabolically inactive persister cells are able to survive antibiotic treatment (17,18). The presence of drug efflux pumps is another attribute facilitating bacterial survival under harsh conditions. A number of hospital-related pathogens such as *Acinetobacter baumannii, Bacillus subtilis, S. aureus* and others exhibit tolerance towards antibiotics due to the overexpression of their drug efflux pumps, and often exhibit a multi-drug resistant phenotype (19).

The unique composition and characteristics of biofilms make treatment of biofilm infections extremely difficult. Apart from the features mentioned above, factors such as genetic variations or cell wall modifying enzymes may also contribute to the increased drug resistance phenotype of biofilms. Moreover, it is now well recognized that many disease-associated biofilms are polymicrobial in nature, leading to synergized tolerance profiles of multiple resistance mechanisms. Therefore, treating biofilms with single-target approaches may not be effective, as distinct, compartmentalized microenvironments with unique characteristics are present within biofilms.

Several approaches, including genetic and phenotypic characterization, have been used for understanding the critical factors regulating biofilm formation and biofilm drug tolerance of microbes (8). The complex changes governing the biofilm state require multi-faceted, systems-level approaches to elucidate the reprogramming of microbial functions in this phenotype. Advances in 'omics' tools such as transcriptomics, proteomics, metabolomics and the integration of multi-omics data hold significant promise in investigating the systems-level regulation of biofilms. Gaining better perspectives on the biofilm phenotype of microorganisms can enable the development of effective strategies that can control both clinical and environmental biofilms. This review provides an overview of how different omics techniques such as transcriptomics, proteomics and metabolomics have been employed in microbial biofilm studies and the application of multi-omics approaches towards obtaining a holistic picture of the biofilm formation process.

Transcriptomics-first-stage biofilm monitoring tool

Transcriptomics is the study of the transcriptome, which comprises the total mRNA transcripts produced by the genome (20). The transcriptome contains coding RNAs that dictate the composition of the proteome; therefore, studying an organism's transcriptome can provide a first-level biochemical picture of how genes are regulated under specific conditions such as transition from planktonic to biofilm state and drug treatment (21,22).

The analysis of the transcriptome can be performed by traditional approaches such as quantitative polymerase chain reaction (qPCR) or high-throughput approaches such as microarray and RNA-sequencing (RNA-Seq) (20). The qPCR method allows real-time monitoring of gene expression and can be used to detect gene expression changes in biofilm state or after exposure to compounds, for example, *esp* gene expression after zinc oxide nanoparticle treatment in *Enterococcus faecalis* biofilms (23). qPCR can also be used to confirm or validate microarray/RNA-Seq results (24), and although this method is rapid and relatively low cost, it can target only known sequences and only few targeted genes can be investigated.

DNA microarrays are the next most commonly used tool after qPCR in transcriptomics studies. Collections of targeted DNAs embedded within wells on a chip enable DNA microarrays to examine the expression levels of numerous genes simultaneously (25). In a recent transcriptomic study, Ebersole *et al* (26) used DNA microarrays to compare the immune response of oral epithelial cells to infections caused by multispecies oral pathogenic bacteria in planktonic and biofilm state. Microarrays are also relatively low cost; however, the scanner is limited to a low dynamic range. With the increasing availability of next-generation sequencing (NGS) platforms, the popularity of DNA microarrays is declining and will soon be futile.

RNA-Seq using NGS is now a popular high demand choice for the detection and quantification of both known and novel transcripts. It exhibits a higher capacity for detection of gene targets as well as a higher sensitivity than microarrays, with single-base resolution for quantification of rare variants and transcripts (20). Although NGS platforms are relatively costly and labor-intensive, the comprehensive results obtained from RNA-seq make it ideal for gene expression studies. For instance, Cheng *et al*. used an HiSeq 2500 sequencer to study the transcriptomics responses of *P. aeruginosa* interaction as the dominant species in a microbial community (27). Additionally the throughput of these technologies continue to increase as the cost per/reads

continues to reduce allowing the technology to be adopted by an increasing number of research groups.

Due to the wide availability of transcriptomic tools, several microbial biofilm studies have employed transcriptomics approaches to study processes such as biofilm development, biofilm-host interactions, and biofilm drug resistance mechanisms. A list of studies on the biofilm transcriptome of medically important microorganisms to address biofilm resistance, lifestyle differences between planktonic and biofilm cells, biofilm-host interaction, and other traits is provided in Supplementary Table 1.

Transcriptomic strategies to uncover drug resistance of microbial biofilms

High-throughput transcriptomic tools have been employed to better understand the drug resistance of microbial biofilms. Particularly, many of the recent studies have investigated the transcriptomic profiles of drug-treated vs untreated biofilms by RNA-seq analysis. For instance, an RNA-seq analysis by Seneviratne et al (28) revealed genes associated with *E. faecalis* biofilm formation and drug resistance. The study identified putative association of sporulation regulatory gene *paiA*, arsenic resistance operon genes *arsR* and *arsD*, ATP-binding cassette drug transporters, and penicillin-binding proteins with biofilm drug resistance. Similarly, Liu *et al.* (29) performed an RNA-seq analysis on the effect of a low ampicillin concentration on *S. aureus* biofilm formation. The ampicillin-induced biofilms exhibited more viability and significantly higher expression of genes encoding penicillin-binding proteins, multidrug resistance efflux pumps, and antimicrobial resistance proteins, indicating a positive response to ampicillin treatment. Wu et al (30) performed RNA-seq analysis of the response of methicillin-resistant *S. aureus* (MRSA) to *G. chinensis* aqueous extract. The expression of biofilm formation and carbohydrate metabolism-related genes was significantly downregulated in MRSA treated with *G. chinensis*. Differential regulation of multidrug efflux pumps and penicillin-binding proteins was observed under drug treatment conditions across most of these studies, indicating their role in mediating microbial drug resistance mechanisms. However, results from these studies require further validation by targeted molecular studies or other omics tools, as the transcriptome is still subject to variations at the translational and post-translational level.

Transcriptomic studies of oral biofilms

Transcriptomic analysis of clinical samples such as saliva and plaque can help in understanding the gene expression changes resulting in dysbiotic microbiome signatures associated with periodontal diseases, dental caries, and persistent root canal infections (31,32). In-depth studies of the gene expression changes associated with dysbiotic microbiome could be the precursor to the development of drugs and treatments that can reduce the prevalence of prolonged infection among dental patients. For example, Yost et al (32) conducted a metagenome and metatranscriptome (RNA-seq) analysis of subgingival biofilm samples from patients with periodontitis. The study identified distinct molecular signatures linked to the progression of periodontitis. In addition, the study observed that various TonB-dependent receptors, aerotolerance genes, proteases, peptidases, hemolysins, iron transport genes, and CRISPR-associated genes were upregulated by the periodontal pathogens *Porphyromonas gingivalis* and *Tannerella forsythia*. Similarly, a study by Duran-Pinedo et al (33) conducted a metagenome and metatranscriptome analysis on the subgingival biofilm samples of patients with and without periodontitis. The study identified that biological processes related to iron acquisition, lipopolysaccharide synthesis, and flagellar synthesis were overexpressed in disease state. The periodontitis samples also had higher mean abundance of *P. gingivalis*, *T. forsythia* and *Treponema denticola* . In both studies, the majority of virulence factors upregulated in patients with periodontitis were associated with organisms that are not usually considered major periodontal pathogens. This suggests that changes in the overall oral community rather than a few pathogens can cause increased virulence activity, which leads to disease progression. It is also noteworthy that both of these studies conducted metagenome and metatranscriptome analysis together to directly correlate the metabolic activities profile with the dysregulated oral microbiome. This is an extremely insightful approach and when coupled with other omics tools such as proteomics can greatly further our understanding of the dysbiotic oral microbiome.

Transcriptomics studies on biofilm development and maturation

Adhesion and successful colonization of solid surfaces by microbes play key roles in biofilm formation. The initial adhesion and colonization of microbes may differ depending on the nature of the surface (34). A comparative DNA microarray-based transcriptomic analysis by Shemesh et al (35) was used to identify the differential gene expression in *S. mutans* biofilms based on biofim thickness. Comparative transcriptome analysis was performed for biofilm

depths of 100, 200, and 400 microns. Analysis of the differentially regulated genes indicated that WapA, a major surface protein, was upregulated in the 200 and 400 microns biofilm compared with the 100 microns biofilm. Peterson *et al.* (36) performed a transcriptomics study of biofilm formation within dental plaque using RNA-seq, which provided unique insights into biofilm biochemical properties, in addition to environmental and genetic factors that influence dental plaque biofilm formation. RNA-seq analysis allowed mapping of metabolically active members of the dental plaque community to 27 genera, with *Streptococcus* spp being the most abundant, followed by *Veillonella* spp and *Capnocytophaga* spp. Further, correlation analysis indicated that *Streptococcus* spp. exhibit weak positive correlations with one another. Functional mapping of the transcripts showed that the most abundant transcriptional categories belong to protein translation and carbohydrate utilization.

A study by Sztukowska et al (37) investigated the role of the internalin-family protein InlJ in mediating inter-species adhesion between *Candida albicans* and *Porphyromonas gingivalis* by mutagenesis and RNA-seq analysis. Transcriptional profiling established that 57 genes were uniquely upregulated in an InlJ-dependent manner in *P. gingivalis*-*C. albicans* communities, including components of the type IX secretion apparatus. The results indicate that *C. albicans* could potentially increase the pathogenicity of *P.gingivalis* through induction of the type IX secretion system. This induction of the type IX secretion system could also be a possible defense strategy employed by *P. gingivalis* against *C. albicans*. A study on the type VI secretion system of *Serratia marcescens* showed that antifungal effectors are deployed via the type VI secretion system against microbial competitors such as *C. albicans* in a coculture of *S. marcescens* and *C. albicans* (38).

Single-cell transcriptomics

The majority of the current transcriptomic studies are performed under the assumption that the biofilm population is homogenous. However, biofilms comprise a heterogenous population of cells with varying metabolic activity levels, which may dictate the antimicrobial resistance profile observed in biofilms. Therefore, for such cases, analyzing the transcriptomic profile at the single cell level can provide a more detailed insight into the specific factors mediating antibiotic/drug resiatnce in microbial biofilms. With the advances in next-generation sequencing, it is possible to elucidate the transcriptome profile at the single-cell level. For instance, a study by Peyrusson et al (39) performed an in-depth RNA-seq analysis of *S. aureus*

persisters that were induced by prolonged antibiotic exposure in a macrophage infection model. Trascriptomic analysis indicated that overall metabolic activity levels were downregulated, including those associated with proliferation and amino acid metabolism. However, the persisters remained metabolically active with transcriptomic reprogramming. Pathways associated with central carbon metabolism were adjusted to redirect transcription towards adaptive responses.

Collectively, transcriptomic approaches provide an opportunity to more fully comprehend the key biological processes driving biofilm formation, drug resistance, etc as opposed to investigating a limited number of preconceived genes of interest. However, the nature of the model (in vitro or in vivo) used for transcriptomic analysis may influence the gene expression patterns. A study by Cornforth et al (40) performed RNA-seq analysis of *P. aeruginosa* from three different sample types, namely, in vitro cultures, human infections, and mouse infections. Machine learning approaches were used to identify the transcriptome signature that can distinguish between human infection and in vitro cultures of *P. aeruginosa*. This transcriptome signature included many of the genes from central carbon metabolism and iron acquisition.

As mentioned earlier, transcriptomics represents only the first stage in gene expression. Numerous changes occur at the translational and post-translational level, which can ultimately lead to phenotypes that may or may not reflect the transcriptomic profile observed. The data observed at the transcriptomic level may therefore not show perfect correlation with higher level omics data such as proteomics or metabolomics data. Hence, data derived from transcriptomic studies should be treated with caution and should be substantiated by next level omics tools such as proteomics and metabolomics.

Proteomics – second-stage biofilm monitoring tool

The term “proteome” represents the entire set of proteins expressed by an organism at a given point of time under a defined set of conditions (41). While the transcriptome is usually a reflection of changes at the gene level, monitoring the proteome profile can provide a more holistic and stable picture of the biological changes occurring within an organism. Moreover, microbes can sense stress/harsh environmental conditions and accordingly adjust their protein expression to overcome the challenges they are exposed to (42).

Proteomics encompasses qualitative as well as quantitative analysis of the proteome. It enables the accurate analysis/measurement of changes in organisms under growth, development, and other environmental conditions. Early proteomics studies used gel-based proteomics approaches (43). However, in recent years, gel-free approaches have become more widespread due to their simpler sample preparation steps as well as advances in mass Spectrometry (MS) techniques (44,45). MS-based techniques enable identification and quantification of a larger number of proteins in a relatively short period, including low-abundance proteins and difficult protein fractions such as membrane proteins. Differential protein expression can be quantified by label-free or isotopic labeling MS methods. Both freeware and commercial software packages are available for proteomics data analysis. APEX (46), Census (47,48), MapQuant (49), MaxQuant (50), MaXIC-Q (51), and MSQuant (52) are some of the freeware for proteomics data analysis, while ProteinPilot, Pro Quant, BioWorks, Elucidator, and Progenesis are commercial software (53).

Proteomics tools used in biofilm studies

The use of high-throughput proteomics has generated a great wealth of information, and it is now being used extensively to study microbial biofilms (54). A list of studies on the biofilm proteome of medically important microorganisms to address biofilm resistance, phenotypic differences between planktonic and biofilm cells, biofilm-host interaction, and other traits is provided in Supplementary Table 2.

Gel-free, differential isotopic labeling methods such as Isotope-Coded Affinity Tags (ICAT), Stable-Isotope Labeling by Aminoacids in Cell culture (SILAC), isobaric Tags for Relative and Absolute Quantitation (iTRAQ), and Tandem Mass Tag (TMT) have been used in many of the recent microbial biofilm studies for relative quantification of proteins (55–58). For instance, Philips et al (59) used SILAC labeling approach for proteome profiling of planktonic vs biofilm *Neisseria gonorrhoeae*. Similarly, other groups have investigated the differential expression profile of biofilms comprising both antibiotic sensitive and resistant strains of *Aeromonas hydrophila* and *Candida* biofilms by TMT labeling and iTRAQ analysis, respectively (60–63).

Label-free methods are based on counting unique spectra/peak intensities and have also been used in many of the biofilm studies, including both bacterial and fungal biofilm studies (64–71). For instance, Bao et al employed a label-free, quantitative proteomics approach to study

the interaction of *A. actinomycetemcomitans* with other oral bacteria in a 10-species subgingival biofilm model (67). In the presence of *A. actinomycetemcomitans*, all of the quantified proteins of *Prevotella intermedia* were found to upregulated, whereas most of the quantified proteins from *S. anginosus*, *Campylobacter rectus*, and *P. gingivalis* were downregulated. Pathway enrichment analysis showed that the differentially regulated proteins were related to 5S RNA binding capacity, ferric iron binding, and metabolic rate.

Proteomics studies on biofilm-host interaction

To date, only a limited number of studies have investigated host-biofilm interaction using proteomics. One particular study, performed by Bostanci et al (66) used label-free proteomics to profile the proteins secreted by the host in a co-culture model of multi-layered gingival epithelium interacting with a 10-species subgingival biofilm. Overall, the number of proteins secreted by the gingival epithelium was reduced when interacting with the biofilm. Inflammation and apoptosis pathways were upregulated in the gingival epithelium, whereas pathways associated with the disruption of epithelial tissue integrity and impairment of tissue turnover were downregulated. Further negative regulation of metabolic processes and degradation of various molecular complexes was observed over 48 h. The study identified a key role of the "red-complex" bacterial species in the mediation of the host immune response.

Proteomics studies on biofilm drug resistance

One of the most intriguing traits of microbial biofilms is their increased ability to withstand harsh environmental conditions such as antimicrobial challenges, in comparison with planktonic cells. Proteomics studies have provided valuable insights into the elusive mechanism of increased resistance in microbial biofilms.

Biofilms comprise a heterogeneous collection of cells with varying metabolic activity levels. The increased resistance of microbial biofilms can possibly be attributed to the slower rates of growth, protein synthesis and metabolic activity observed within biofilm communities. For instance, a shotgun proteomics study on *E. coli* biofilms observed that energy generating pathways such as glycolysis and pentose-phosphate pathways show lower expression levels in biofilms (9). Similarly, decreased expression of glycolysis pathway has also been observed in *S. mutans* biofilms vs planktonic proteome (72). A study on *Candida glabrata* biofilms observed decreased expression of carbohydrate metabolism pathways in biofilms vs planktonic

cells (73). Proteomics analysis of *A. fumigatus* biofilms has shown that metabolic activity decreases as the biofilm matures, with lower expression of enzymes of ATP synthesis, TCA cycle, and glycolysis (74). However, some studies have provided contradictory views showing similar or higher metabolic activities in the biofilm state compared to planktonic state (75,76).

Another possible hypothesis accounting for the increased drug resistance in microbial biofilms is the activation of oxidative stress response pathways. Proteomics studies on *Candida* biofilms have observed that biofilms show enhanced anti-oxidative capacity. Anti-oxidant proteins such as Ahp1p and Trx1p are expressed at higher levels in the biofilms of *C. albicans* and *C. glabrata* compared with plaktonic cells (73,77). Similarly, a proteomics study on Shiga toxin-producing *E. coli* O157:H7 (STEC) biofilms demonstrated higher expression levels of periplasmic antioxidant systems (SodC and Tpx) in the biofilm vs planktonic phenotype (78). In a proteomics analysis of biofilm vs planktonic *Neisseria meningitidis,* oxidative defense proteins were expressed at higher levels in the biofilm vs planktonic state (79). In another study, increased expression of stress response proteins including antioxidants was observed in the *Actinomyces naeslundii* biofilm vs planktonic proteome (80). Similarly, higher levels of anti-oxidant proteins were observed in *P. aeruginosa* in the biofilm state (81). *Listeria monocytogenes* and *Salmonella enterica* have also shown higher expression levels of superoxide dismutase in the biofilm vs planktonic mode, indicating higher anti-oxidative capacities in the biofilm state (82,83). The above studies support the role of anti-oxidant defense system in mediating increased antimicrobial resistance of biofilms (57).

Some biofilm proteomics studies have characterized the differential protein expression profiles in strong and weak biofilm forming strains to elucidate the markers regulating the extent of biofilm formation. A comparative proteomics study of strong vs weak biofilm forming *E. faecalis* strains by Qayyum et al (84) showed that weak biofilm formation is associated with downregulation of the osmotically inducible protein C, an OsmC/Ohr family oxidative stress protein. Another study by Suriyanarayanan et al (61) on strong vs weak biofilm forming *E. faecalis* clinical isolates identified lower levels of metabolic activity in the strong biofilm former relative to the weak biofilm former, signifying the role of metabolic activity levels in governing biofilm formation.

Thus, the theme of metabolic activity levels governing biofilm formation as well as the extent of biofilm formation has been well explored in several proteomics studies. Although the results from some of these studies have been contradictory, it is worthwhile examining the metabolic

activity levels of persister cells to glean a better understanding of the role of metabolic activity levels in regulating biofilm formation.

Proteomics studies on biofilm EPS

Many studies have examined the factors associated with increased drug resistance in microbial biofilms. There are differing opinions within the literature on the importance of the extracellular matrix and the polysaccharide components in drug resistance. For example, a study comparing the proteome of *S. enterica* under different high- and low-flow conditions demonstrated that there were no major pattern shifts in whole cell protein expression under the different flow conditions, despite some changes in the EPS composition and biofilm structure (83). The majority of EPS studies have looked at their polysaccharide composition; however, some are specifically targeted toward their protein composition. The study of the EPS polysaccharide components is better suited for metabolomics (85–87). The complex nature of the EPS, which is conferred protection by several components, complicates the recovery of EPS proteins. This presents significant challenges in studying the EPS proteins, particularly in getting a reproducible protein extraction procedure. Physical and chemical extraction and a combination of both methods have been used to extract EPS. Therefore, for the most efficient protein recovery, it is important to optimize the extraction methodology prior to the beginning of the experiment.

An exoproteome study by Gil et al (88) on exopolysaccharide- and protein-based biofilm matrices of methicillin sensitive and methicillin-resistant *S. aureus* and a clinical strain showed that *S. aureus* expressed higher levels of immuno-evasive proteins in the biofilm vs planktonic cells. In another analysis of EPS proteins obtained from *P. aeruginosa* biofilms at different stages, presence of putative type III secretion system effectors was observed in the matrix, suggesting that ECM proteins may have roles in stress resistance, nutrient acquisition and pathogenesis (89).

A study on *C. albicans* EPS by Zarnowski et al (90,91) showed that proteins comprise the majority of the EPS, far exceeding the polysaccharide content, contrary to common belief (90). However, proteomics technologies have not yet been fully utilized in the direction of studying EPS components of microbial biofilms.

Proteomic profiling of drug response in biofilms

Drug resistance is one of the major phenotypes attributing to the persistence of the biofilm mode of growth. The drug resistance phenotype in biofilms has been investigated in a wide array of conditions. A proteomics study on the biofilms of chlortetracycline-resistant and susceptible *A. hydrophila* strains showed that increased fatty acid biosynthesis may be associated with antibiotic resistance of *A. hydrophila* (60). In another study examining the inhibitory effects of carolacton (secondary metabolite isolated from the myxobacterium *Sorangium cellulosum*), a disturbance in peptidoglycan biosynthesis and degradation was observed in both planktonic and biofilm cells, suggesting that carolacton results in cell death by damaging the cell wall integrity (92).

Some proteomics studies have specifically examined the proteome of the persister population to understand the drug resistance process. A study on *P. aeruginosa* biofilm persisters demonstrated increased expression levels of type IV pili assembly proteins and quorum sensing-regulated proteins such as chitinase, LasB, and phenazine/pycocyanin synthesis proteins in the persister subpopulation (58). The study suggested that type IV pili aids in the migration of antibiotic-tolerant cells to the top layer of biofilms, with new antibiotic-tolerant subpopulations established with the help of quorum sensing. The study proposed that the use of motility and quorum sensing inhibitors along with traditional antibiotics could prevent persistent infections.

Metabolomics – end-point biofilm monitoring tool

Metabolites are low molecular weight intermediates or end-products of enzyme-catalyzed reactions in a cell. Metabolites can be classified into two types, namely, primary and secondary metabolites. Primary metabolites are vital for cell growth and development and are produced actively during growth phase (e.g. amino acids and ethanol). Secondary metabolites are not essential for growth and development but may have specialized roles in ensuring overall survivability and adaptability (e.g., antibiotics and second messenger signalling molecules) (93). Metabolomics is the comprehensive detection, identification, and quantification of the metabolome of a biological system. The profiling of metabolites can provide insights into the physiological, pathological and biochemical status of a cell. Metabolomics is an end-point monitoring tool, as metabolite profiles can be directly correlated with the observed phenotype unlike genes and proteins. Genes or proteins may undergo epigenetic or post-translational

modifications, and hence the observed transcriptomic or proteomic profiles may not be directly reflective of the observed phenotype. In some cases, protein or gene expression changes may affect the metabolite levels even in the absence of obvious phenotypic changes.

Biofilm metabolomics

Metabolomics is a valuable tool for studying antimicrobial resistance in biofilms (94). Key primary and secondary metabolites can influence biofilm formation significantly; therefore, metabolome profiling, coupled with modeling of biological systems, can facilitate the identification of pathways associated with biofilm formation. This, in turn, can enable new strategies for controlling biofilm formation and development. The metabolite flux observed during drug/antibiotic resistance can point to the key molecules/mechanisms mediating this process, leading to the development of targeted therapeutics against the identified molecules. For instance, metabolomics tools have been employed to identify *S. aureus* antibiotic resistance mechanisms (95). Similarly, the effect of different environmental stimuli on biofilm formation has also been investigated by metabolite changes monitoring (96). Drug-discovery studies can also benefit significantly from metabolomics approaches. For example, metabolomics screening of molecules that inhibit or mimick the activity of autoinducers, which have crucial roles in biofilm formation, is a promising approach to combat biofilm formation (97). Metabolomics identification of intra- or intercellular small molecules associated with biofilm formation and development can also lead to potential drug targets (98).

Biofilms comprise a spatially organized, heterogeneous collection of cells with varying states of metabolic activities to maximize survival. To better understand the nature of biofilms, a comprehensive analysis of all the metabolic states that occur within this intricate community is essential. Metabolomics offers a systematic platform to analyze the complex biofilm community (98).

Metabolomic approaches for studying biofilms

Microbial systems are ideal for performing metabolomics studies, as they can be easily manipulated. However, the observed number and types of metabolites can differ dramatically in different organisms, rendering the technology less generalizable and necessitating organism-specific optimization (99,100). Moreover, the complex inter-networking of metabolic

pathways and their sensitivity toward even minor changes in the system present considerable challenges in obtaining an overall metabolite fingerprint. Additionally, a wide range of compounds, including volatile alcohols, hydrophilic carbohydrates, amino /non-amino organic acids, hydrophobic lipids, ketones, and complex natural compounds are observed in a metabolome (101). The use of a single approach may not always be sufficient to profile all these compounds due to their distinct properties and may require complementary analytical platforms. The most common analytical techniques used in metabolomics are MS- and nuclear magnetic resonance spectroscopy (NMR)-based platforms (102,103). A list of metabolomics studies of medically important microorganisms to address biofilm resistance, phenotypic differences between planktonic and biofilm cells and other traits is provided in Supplementary Table 3. Detailed information on the analytical platforms used for metabolomics analysis has been outlined in greater detail in previous articles (104–107). Metabolite profiling can be performed via either untargeted or targeted approaches (108,109). The overall goal of the study and number of metabolites targeted are some of the factors governing the choice of a suitable metabolomics approach.

Untargeted metabolomics

Untargeted or global metabolomics approaches do not attempt to identify or precisely quantify all the metabolites in the sample. A snapshot of the metabolite profile occurring at a particular state/time point is captured via this approach. Untargeted approaches are especially suited for drug discovery or other discovery-based studies (110). The aim of untargeted metabolomics is to capture as many metabolites as possible in the samples under comparison without any bias and is usually implemented as a hypothesis-generating approach. Monitoring of global metabolic profiles occurring under different physiological states (planktonic vs biofilm) (111,112) or drug-treatment/stress conditions (113) would provide an overview of the changes occurring within a system and can provide key insights into the regulatory pathways mediating bacterial lifestyle switch, drug resistance, or persister cell formation. However, there are many technical difficulties associated with untargeted metabolomics, which may lead to bias in analysis. The wide range of polarities of the metabolome makes total extraction difficult, necessitating the implementation of various extraction techniques to reduce bias at the extraction stage. Other technical limitations include sensitivity issues for compounds with low detection limits and usage of certain ionization modes, chromatography columns and buffers

in mobile phases, leading to better separation or ionization of particular sets of metabolites. The inherent limitations of MS scanning make it easier to detect metabolites at higher concentrations than those at lower concentration. In addition to the above, missing data and statistics make the dataset generated from an untargeted metabolomics experiment significantly more complex, hindering the interpretation of meaningful data (114). Since manual inspection of metabolomics data is difficult, specific software have been developed for data analysis. Both freeware and commercial software are available for data preprocessing and analysis. MetDAT, Mzmine, MetAlign, XCMS, MetaboAnalyst, MetExplore, MathDAMP, and datPAV are examples of freeware, while Mass Profiler Professional (MPP), Mass Hunter, Progenesis, and Sieve are examples of manufacturers' proprietary software (115–121). Although these software products have contributed hugely towards the elucidation of significant metabolite patterns, tools for further validation are required for successful interpretation of untargeted metabolomics data (122). Untargeted metabolomics can nevertheless be a valuable drug discovery tool and can aid in the identification of novel biofilm formation pathways (108,123).

Targeted metabolomics

Targeted metabolomics approaches are driven by a specific hypothesis or biological question. For example, the targeted approach is an appropriate tool in studies aiming to quantify specific polysaccharides of the biofilm EPS matrix (124). Since targeted approach probes specific pathways of interest, it can provide a direct answer to the biological question under study. Targeted metabolomics can be used to directly quantify metabolites of interest. This approach is extremely sensitive and allows for very low limits of detection (124). Targeted metabolomics approaches are usually performed as a follow-up to either untargeted metabolomics or other omics approach such as proteomics (125). This is because targeted analysis usually requires a priori knowledge of the metabolite of interest generated from a more global approach first (126). To perform targeted analysis, a metabolite standard for the metabolite of interest should preferably be available in a purified form. While the targeted approach cannot be used to discover novel compounds (127), quantitation of the metabolite of interest and novel associations of known compounds to pathways can still be made, making it a useful technique to be used together with untargeted metabolomics.

Metabolic flux analysis

While metabolomics provides a snapshot of the metabolome at a certain state, metabolic flux analysis (MFA) enables monitoring of the metabolome over time. In MFA, stable or radioisotopes of primary or secondary metabolites are used in the bacterial culture to produce downstream intermediates of the metabolic pathway. Stable isotopes do not require the additional safety precautions of radioactive work, and the metabolite flux can be monitored using mass spectrometers. Mathematically, the changes of intracellular metabolite pools are expressed as functions of reaction stoichiometries and fluxes to and from these pools in MFA (128). MFA helps to overcome the limitations of conventional metabolomics by enabling the tracing of pathways of labelled metabolites. MFA also highlights the importance of changes in metabolite turnover rather than that of changes in concentrations, which might not be reflected in static metabolomics.

In a study of *P. aeruginosa* biofilm formation by Wan et al (129), significantly different metabolic pathways between biofilm and planktonic cells were discovered by 13C tracing. The turnover of labelled glucose-6-phopsphate was slower in the biofilm, indicating slower metabolism. In another study of *Corynebacterium glutamicum* by Krömer *et al* (130), cultures of the bacteria in stable isotopes of glucose showed increased flux through the lysine production pathways and TCA cycles, but these were not reflected in transcriptional or protein level changes. This further illustrates the need for a multi-omics approach when studying the metabolic changes of bacteria.

Challenges in biofilm metabolomics

As mentioned earlier, the number and types of metabolites observed vary based on microorganisms. In some cases, the identity of compounds observed maybe unknown. Additionally, the metabolic products generated are constantly in a state of flux and are highly variable in their chemical structure and properties. Dynamic changes in metabolite levels make it difficult to capture the complete snapshot of metabolite profile at a given physiological state. In the context of biofilm metabolomics, the variability in EPS matrix composition and the interactions among its various components add new layers of complexity, posing considerable challenges towards the development of EPS-targeting therapeutics. Sample extraction is a crucial step that must be considered carefully as it determines the success of any metabolomics experiment. The ideal sample extraction strategy should ensure that metabolites across all

classes of compounds are represented (131,132). This is usually ensured by separating the metabolite pool into organic and hydrophobic phases and analyzing them separately.

The composition of EPS, which is one of the major biofilm components determining the resistance behavior, complicates both the extraction and analysis process (133). Exopolysaccharides comprise the majority of the EPS. While some of the exopolysaccharides are homopolysaccharides, most of them are heteropolysaccharides comprising a mixture of charged and neutral residues. Alginate, cellulose and poly-N-acetyl glucosamine (PNAG) are some of the well-known bacterial biofilm exopolysaccharides (133,134). Alginate consists of the uronic acid residues β-d-mannuronate (M) and its C-5 epimer, α-l-guluronate (G). It provides mechanical stability to the biofilms and is involved in microcolony formation in the early biofilm development stages (134). Cellulose is a polymer consisting of β-1-4–linked linear glucose chains. It is a crucial component of the ECM of Salmonella and *E. coli* biofilms (133). PNAG is a linear homoglycan comprising β-1, 6-N-acetylglucosamine residues. It has essential roles in adherence and biofilm formation of certain bacterial species (133). β-1,3-glucans and galactosaminogalactan are some of the common exopolysaccharides found in fungal biofilm matrix. β-1,3-glucans helps in sequestration of antifungal agents and prevents recognition by neutrophils in *C. albicans*. Galactosaminogalactan mediates adhesion to surfaces and confers protection against host responses in *A. fumigatus* (135).

Many of the extraction methodologies for EPS are suitable for their soluble portion only (134). The EPS has an abundance of carbohydrate moieties, most of which are insoluble and difficult to separate from the cells. This complicates the extraction process and very few methodologies keep this criterion in consideration. Moreover, the EPS polysaccharides can be in either ordered or disordered forms, with disordered forms being favored by elevated temperatures and low ionic concentrations (136). The exposure of biofilms to different hydrodynamic conditions based on the surrounding environment can influence the biofilm matrix composition and structure. The ECM also consists of proteins, DNA, and lipids (133). A single extraction methodology may not be ideal to obtain all the EPS metabolites; therefore, a multi-method protocol encompassing a wide spectrum of biological macromolecules is required. Further, it is difficult to obtain a proper balance between comprehensiveness and metabolite stability, as extraction conditions that favor preservation of one metabolite class may not be suitable for other metabolite classes. Ultimately, extraction methodology should be geared toward required metabolite range and the specific analytical technique used.

A unique method for overcoming problems with extraction involves the use of a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The method allows the study of intact bacterial cells by directly transferring the cells from agar plates or pelleted cultures and covering with a matrix layer for MALDI-TOF MS analysis (137). For instance, studies by Caputo et al (138) to identify biofilm producer and non-producer of *Staphylococcus epidermidis,* and Santos et al (139) to study proteins of *Listeria monocytogenes* biofilm under different conditions have used this analysis method.

Current applications of biofilm metabolomics

Metabolomics analysis of biofilms has been employed across a number of studies within the literature. These studies use either the targeted or untargeted approach depending on the nature of the question addressed, biofilm model employed, and the complexity of metabolites addressed. Metabolomic approaches have been used to address a wide range of biofilm phenotype-related questions. Some studies have used metabolomics to investigate the determinants of the phenotypic switch between planktonic and biofilm state or the differences between the two states. For instance, studies by Gjersing et al (140), Yeom et al (141), Chavez-Dozal et al (142), Stipetic et al (111), Zhang et al (143), Favre et al (144), and Zabek et al (145) investigated the differences between the planktonic and biofilm states in *P. aeruginosa*, *Acinetobacter baumanii*, *V. fischeri*, *S. aureus*, *Desulfovibrio vulgaris*, *Pseudoalteromonas lipolytica*, and Aspergillus respectively. While most of these studies used an untargeted approach, targeted approach was used specifically by Zhang et al and Chavez-Dozal et al.

Several studies have employed metabolomics approach to study mixed-species biofilms. Mixed-species biofilm studies by Sasaki et al (146), Adamiak et al (147), and Weidt et al (148) focus on mixed species biofilms in the context of anodic biofilms, halophilic biofilms, and a mixed species biofilms of *C. albicans* and *S. aureus*, respectively. Other mixed-species biofilm studies are focused on biofilms formed in the oral milieu. For instance, studies by Agnello et al (149), Califf et al (150), and Edlund et al (151) use metabolomics to investigate in-vitro mixed-species biofilm models obtained from saliva and the effect of sodium hypochlorite treatment on biofilms in periodontal pockets. A study by Slade et al (152) has employed targeted metabolomics approach to investigate *P. aeruginosa* in a collagen wound biofilm model.

Some of the metabolomics studies evaluate the response of biofilms to stress conditions. Studies by Favre et al (113) and Booth et al (153) investigate the differential response of

planktonic vs biofilm cells in response to copper exposure in *Pseudoalteromonas lipolytica* and *P. flourescens*, respectively.

Some studies have addressed very specific questions using metabolomics. For instance, Wong et al (154) have addressed the mechanisms behind the differential biofilm capabilities of *H .pylori* strains using an untargeted metabolomics approach. This study highlights the potential use of metabolomics to not only investigate the reasons behind bacterial lifestyle switch but also the influence of metabolite markers in determining the extent of biofilm formation. Similarly, a study by Zandona et al (155) employed untargeted metabolomics approach to investigate the dental biofilms of caries-free vs caries-active individuals.

Although only a limited number of metabolomic studies have been conducted on biofilms so far, the diversity of applications existing in the current studies signify that metabolomics is a versatile tool for addressing a wide variety of biofilm-related questions. However, it should also be noted that currently there are no metabolomics studies targeting the persister subpopulation of biofilms. This is probably because methods for selectively analyzing the persister cells are still in their infancy and would require considerable advances before being subjected to metabolomics analysis.

The use of metabolomic approaches for studying biofilms is extremely promising because metabolism is highly conserved within biological systems and the analytical approaches used in metabolomics are transferable across different biological systems. Further, recent technological advancements have significantly helped in overcoming the current bottlenecks in biofilm analysis, making metabolomics an invaluable tool in the field of microbial biofilms. The insights on physiological, pathological, and biochemical status obtained from the analysis of metabolites can be further combined with chemical and informatics methods.

Multi-omics approaches and their application in current biofilm research

Single-target or reductionist approaches, as mentioned before, yield limited information in the context of analyzing whole biological systems. With the advent of omics technologies, it has become eminently practical to capture systems-level information of biofilms. The next step towards broadening our understanding of biofilm biology would be to integrate the data available from different omics approaches. This multi-omics or integrated omics approach would provide a holistic perspective of the changes occurring in a biofilm system. However, there are some inherent

628 complexities in a multi-omics data integration approach. First, the sheer volume of data generated
629 is huge, making it computationally intensive to process. Second, the inherent data type is different
630 for each omics approach, thereby complicating the integration process. Therefore, data
631 normalization should be performed before data integration. Data integration usually employs both
632 statistical and machine learning approaches to reduce the dimensionality of data, facilitate clustering,
633 and predict specific markers/features of interest (156). There are several software pipelines now
634 available to facilitate the integration of omics data (157). Web-based tools requiring no
635 computational experience and more versatile tools requiring computational experience are
636 available for omics data integration. Web-based tools that do not require computational
637 experience include 3Omics, Paintomics, and Galaxy (P, M) (156). Tools requiring
638 computational experience include the miodin R package, which allows for vertical data integration
639 (experiments on the same samples) or horizontal data integration (studies on the same variables)
640 (158). mixOmics is another R-based software package that facilitates the exploration and
641 integration of biological datasets with a particular focus on variable selection (159). mixOmics
642 can analyze and integrate omics data such as transcriptomics, metabolomics, proteomics,
643 metagenomics, etc. The software can identify the key features that show high correlation and/or
644 can explain the outcome of interest. IntegrOmics, SteinerNet, and Omics Integrator are also
645 other such computational tools for data integration. Online tools such as XCMSOnline enable
646 integration of metabolomics data with genomics and proteomics data (156). MetaboAnalyst is
647 another online tool that allows integration of metabolomics data with transcriptomics or
648 metagenomics data. A summary of multi-omics study approach is provided in Figure 1.

649 Multi-omics approaches have so far been employed only limitedly in biofilm studies to explore
650 systems-level associations. Most of the existing multi-omics biofilm studies have performed
651 metagenomics and metabolomics analysis together. Multi-omics biofilm studies employing
652 transcriptomics, proteomics, or metabolomics approaches together also exist in the current
653 literature, albeit to a lesser extent. For instance, Harrison et al (160) performed transcriptomics,
654 proteomics, and metabolomics analysis to understand the changes associated with nutrient
655 adaptation and long-term survival of *Haemophilus influenzae*. The study analyzed the
656 transcriptomic, proteomic, and metabolomic profile of 48 h biofilms and identified 29 proteins,
657 55 transcripts, and 31 metabolites that showed significant changes in biofilms under transient
658 heme restriction compared with biofilms continuously exposed to heme-iron. Enzymes in the
659 tryptophan and glycogen pathways as well as adhesin production, metabolite transport, and
660 DNA metabolism were significantly increased under heme restriction. The study identified that

changes in central metabolism coupled with increased stores of nutrients may counterbalance nutrient sequestration. However, in studies conducting multi-omics analysis, the use of multiple time points as opposed to single time points may help to better track the progression from transcripts to proteins/metabolites stage. A study by Favre et al (113) assessed the metabolome and proteome changes induced by copper at growth inhibitory concentrations in the marine bacterium *Pseudoalteromonas lipolytica* under planktonic and biofilm state. Exposure to copper induced defense and detoxification mechanisms, with drastic changes in the lipid composition of the bacterial cell membrane. Moreover, a more heterogenous response was observed in the biofilms compared to the planktonic state under copper stress. A study by Ellepola et al (63) employed transcriptomics and proteomics analysis to decipher the cross-species interaction occurring in *C. albicans-S. mutans* mixed species biofilms. The study revealed a synergistic carbohydrate mechanism in these mixed species biofilms, which promotes co-adhesion. While the above-mentioned studies have used more than a single omics approach to investigate biofilm systems, it should be noted that they have not performed integration of data obtained from the different platforms. With data integration, a better understanding of the direction and dynamics of information flow from genes to metabolites can be obtained.

Future directions of multi-omics based biofilm studies

To date the majority of multi-omics studies have only integrated data obtained from a combination of two omics approaches, eg, transcriptomics plus metabolomics or proteomics plus metabolomics. This may be due to the limitation in data analysis tools that allow only a two-level data integration. However, with improvements in software development and increase in computational power, data integration across three or more levels is now feasible. These significant advancements promise holistic exploration of biofilm biology.

Data integration can be performed via unsupervised or supervised methods (161,162). Unsupervised methods use approaches such as factorization and Bayesian networks to cluster the data into different groups without any external guidance (163). Supervised methods, on the other hand, require external guidance to derive patterns from categorized data such as those based on regression analysis. The derived patterns can then be used on uncategorized data to predict specific outputs (164). Supervised methods can be useful in predicting disease state based on changes in mixed species biofilm composition occurring at specific body niches such as oral or gut biofilms. Interactions between different layers can be a major concern in strategies

for data integration. The corresponding mapping of relationships between different layers such as gene expression to protein expression or to metabolite expression should be considered both independently and together during the integration process (165). Many of the earlier data integration tools did not consider the interactions occurring across different layers of data. They independently performed analysis at each level to find the common subset of biological pathways that are differentially regulated at each layer. However, the more recent state-of-the-art tools such as SNF and iCluster+ consider interactions while integrating different layers. Thus, the internal relationship of different layers is considered as the driving factor that acts in a concerted manner from each omics data (166).

Finally, the availability of multi-omics platforms along with the recent advancements in artificial intelligence (AI)-based tools makes it possible to develop models that can predict pathways/molecular markers associated with specific traits or phenotypes such as drug resistance, biofilm formation, etc. However, this requires large multi-omics datasets collated from existing literature to be deposited in a standardized manner in a global database repository. This database can serve as a training dataset for building models that can associate pathways with biofilm phenotypes of interest. The generated models can then be used to predict pathways or molecular markers associated with biofilm or drug resistance phenotypes in new organisms of interest (Figure 2). The use AI-based integration of multi-omics data has the potential to be immensely useful in drug development strategies as well as for translation of basic research into clinical studies. Integrated multi-omics data can also be combined with other data types, such as electronic health record (HER) data to develop precision medicine.

Disclosure statement

The authors declare no potential conflict of interest with respect to the authorship and/or publication of this article.

Author contributions

CJS and TS, contributed to conception, design, data acquisition and interpretation, drafted and critically revised the manuscript. LSL, ML and JC contributed to design and data acquisition, drafted and critically revised the manuscript. CD and GR, contributed to conception, design and data acquisition, drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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Figure legends

Figure 1. Workflow summary of biofilm multi-omics studies. GC-MS, Gas chromatography mass spectrometry; LC-MS, Liquid chromatography mass spectrometry; NMR, nuclear magnetic resonance; UPLC, Ultra performance liquid chromatography

Figure 2. Proposed workflow for artificial intelligence-based multi-omics prediction platforms. Multi-omics data from existing biofilm studies will be deposited in a global database repository that will serve as a training dataset for developing prediction models. Relevant features selected from the multi-omics database will be subjected to supervised and unsupervised data categorization algorithms to generate prediction models for biofilm phenotypes of interest. The generated model will then be used on multi-omics data generated from new biofilm studies to associate the identified pathways with the corresponding biofilm phenotypes. MS, mass spectrometry; NMR, nuclear magnetic resonance.