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Review Article

Pseudomonas aeruginosa Biofilm Formation: Antibiofilm Strategies and Conventional Methods of Evaluation.

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Abstract: *Pseudomonas aeruginosa* is an opportunistic pathogen known for its ability to form resilient biofilms, which contribute to its persistence in chronic infections and resistance to antimicrobial agents. This review provides a detailed examination of biofilm development stages—attachment, maturation, and dissemination and highlights the role of specific genes in each stage, with a focus on quorum sensing as a key regulatory mechanism governing bacterial communication within biofilms. It also evaluates conventional methods for analyzing *P. aeruginosa* biofilm phenotypes, discussing their purposes, advantages, and limitations. Accordingly, future research should focus on standardizing protocols, advancing imaging technologies, and targeting biofilm-specific mechanisms. Collaborative efforts across disciplines and translational research will be essential for developing effective therapies. This review aims to deepen our understanding of *P. aeruginosa* biofilm dynamics and highlight the implications for developing effective therapies against biofilm-associated infections.

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INTRODUCTION

Biofilms consist of interconnected colonies of bacteria bound in self-excreted matrix known as an extracellular polymeric substance. (EPS) (Vani *et al.*, 2023). These structures aggregate and adhere to the surfaces of medical devices, industrial equipment, and host tissues (such as teeth, gums, wounds, sinuses, bladder, and airways). They interfere with host mechanisms, resulting in health and environmental complications (Shineh *et al*., 2023). Biofilm producing bacteria are major contributors in the development of chronic infection, persistent contamination, and antibiotic resistant (Assefa & Amare, 2022; Dutt et al., 2022).

The common widespread Gram-negative bacterium *Pseudomonas aeruginosa* is well-known for its considerable biofilm-forming capacity and built-in antibiotic resistance., both of which provide serious difficulties in therapeutic settings (Verdial *et al*., 2023). *P. aeruginosa* biofilms may contribute to persistent infections, which are challenging to treat (He *et al*., 2023). These biofilms have been linked to a variety of diseases, such as but not limited to those that impact the urinary tract, respiratory system, and wounds (Sathe *et al*., 2023). These infections are more common in immunocompromised people and cystic fibrosis pateints (Oves *et al*., 2024).

A number of biofilm-associated antimicrobial resistance mechanisms, such as decreased antibiotic penetration, changed biofilm cell metabolic activity, and persisted cell presence, make the conventional antibiotic-based treatment strategy for *P. aeruginosa* infections frequently ineffective (Shrestha *et al*., 2022). As a result, there is increased focus on creating therapeutic agents that specifically prevent the production of biofilms, disrupts already-formed biofilms, or improve the current therapies efficiency (Shrestha et al., 2022).

Research attention have been directed more toward the development of antibiofilm agents in recent years (Damyanova *et al*., 2024). These agents are intended to disrupt established biofilms, prevent biofilm formation, or increase the susceptibility of bacteria associated with biofilms to commonly used antibiotics (Kumar et al., 2023). In recent years, the focus on developing antibiofilm agents has been supported by advancements in several scientific fields, such as screening techniques, molecular biology, and synthetic chemistry (Moreno Osorio et al., 2021).

The objective of this review is to assess conventional methods for identifying antibiofilm agents targeting *P. aeruginosa*. It examines the strengths and limitations of these approaches and explores potential future research directions. By summarizing current knowledge and recent advancements, it may support the development of more effective strategies for combating *P. aeruginosa* biofilm-related infections. Overcoming the challenges posed by these biofilms is essential for improving therapeutic outcomes and advancing our understanding of biofilm biology and antibiotic resistance.

QUORUM SENSING IN *P. aeruginosa*

P. aeruginosa utilizes multiple quorum sensing (QS) systems to regulate gene expression in response to bacterial population density (Smalley et al., 2022). The primary QS systems in *P. aeruginosa* are the Las, Rhl, and Pqs systems (Soto-Aceves et al., 2021). The Las system is one of the most extensively studied quorum sensing systems. It involves the signaling molecule N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), which acts as an autoinducer. The system includes LasI, an enzyme responsible for synthesizing 3-oxo-C12-HSL, and LasR, a receptor protein that binds to this autoinducer to regulate gene expression. The LasR-3-oxo-C12-HSL complex activates transcription of target genes involved in virulence and biofilm formation (Y. Wang et al., 2022). The Rhl system utilizes the AI N-butyryl-L-homoserine lactone (C4-HSL). RhlI produces C4-HSL, and RhlR is the receptor that, upon binding to C4-HSL, regulates genes crucial for producing rhamnolipids and other factors that contribute to biofilm structure and function (J. Li & Zhao, 2020) (Duplantier et al., 2021). The Pqs system is distinct in that it uses the signaling molecule Pseudomonas quinolone signal (PQS) and its precursors (García-Reyes et al., 2020). PqsABCD is involved in PQS biosynthesis, while PqsR (MvfR) is the receptor that mediates the effects of PQS. This system regulates a separate set of genes associated with virulence, biofilm maintenance, and secondary metabolism (Groleau et al., 2020).

P. aeruginosa, quorum sensing is a density-dependent regulatory system that orchestrates bacterial behavior through the production and detection of AIs (Simanek & Paczkowski, 2022). As the bacterial population density increases, the concentration of AIs rises (Eickhoff et al., 2022). These

signaling molecules diffuse freely across the bacterial cell membrane and accumulate in the extracellular environment (Salman et al., 2023). Once the concentration of AIs reaches a threshold level, they bind to specific intracellular receptors, such as LasR and RhlR (Coquant et al., 2020). This receptor-ligand binding activates transcriptional regulators that initiate the expression of genes involved in various processes, including biofilm formation (Zhou et al., 2020; Shao et al., 2020). By coordinating the production of extracellular polymeric substances (EPS) and other biofilm-related factors, quorum sensing ensures that the bacteria form robust biofilms at high cell densities, enhancing their ability to adhere to surfaces, resist environmental stresses, and evade antimicrobial treatments (Singh et al., 2021).

***P. aeruginosa* BIOFILM FORMATION**

The formation of biofilms proceeds through a highly regulated process involving distinct stages: initial attachment, microcolony formation, maturation, and dispersion (Figure 2) (Haidar et al., 2024). At each stage, bacterial cells undergo phenotypic changes facilitated by QS systems, small regulatory molecules, and environmental cues (Ostovar & Boedicker, 2024). QS enables *P. aeruginosa* to synchronize gene expression within the biofilm, regulating virulence factors, EPS production, and antibiotic tolerance (Hemmati et al., 2024; Juszczuk-Kubiak, 2024). The stages of biofilm formation, along with the factors, structures, and conditions that facilitate each phase, are detailed in Table 1.

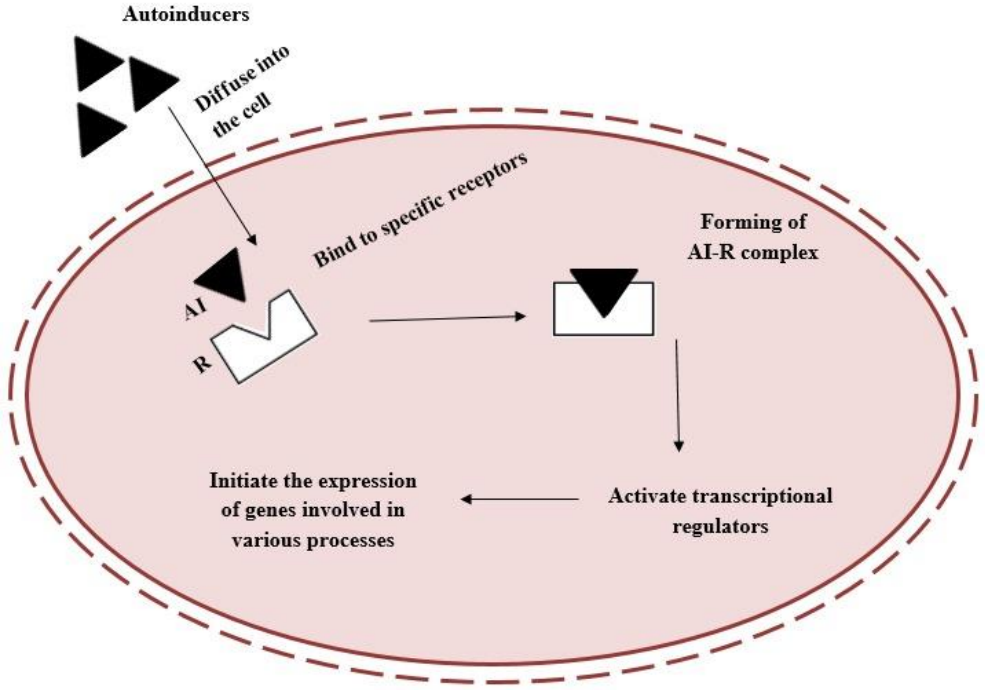

Figure 1: Mechanisms of quorum sensing: from autoinducer accumulation to gene expression

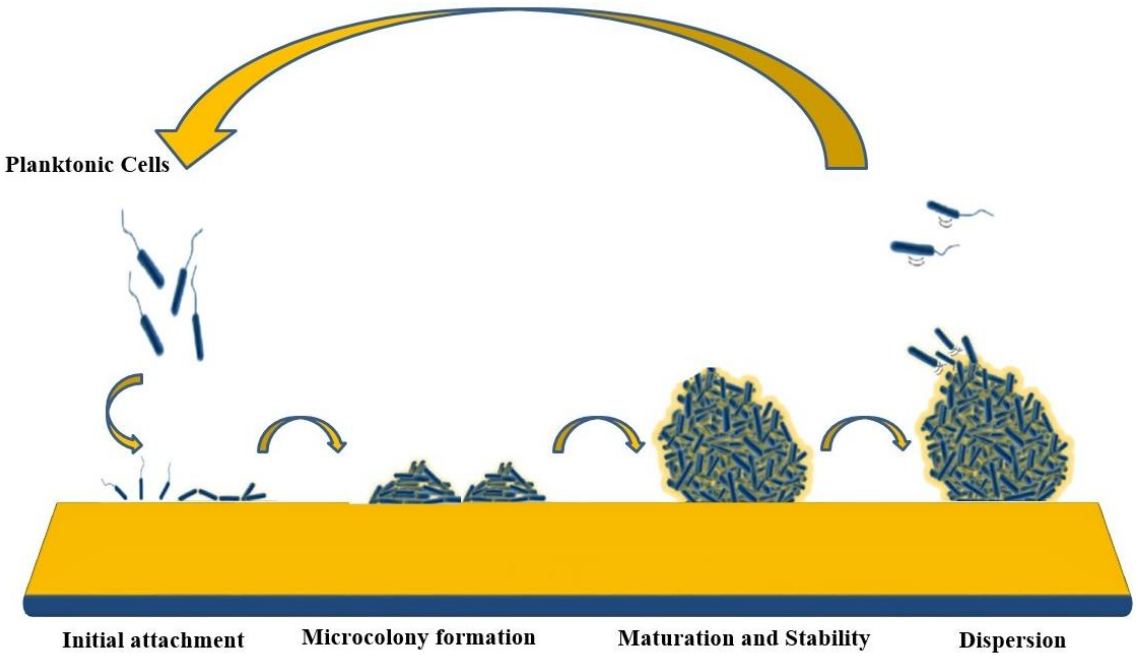

Figure 2: Stages of biofilm formation

Stage I: Initial Attachment

Biofilm formation begins with the initial attachment of planktonic (free-floating) bacterial cells to surfaces (Sharma et al., 2023). This crucial step marks the onset of biofilm development and sets the stage for subsequent stages (Aboelnaga et al., 2024). The gene regulation during the initial attachment stage of *P. aeruginosa* biofilm formation is regulated by a sophisticated network of regulatory pathways (Bai et al., 2021). QS systems (Las and Rhl), type IV pili (Tfp), twitching motility, EPS production pathways, and environmental adaptation mechanisms collectively control the expression of genes involved in adhesion, colonization, and biofilm initiation (Kuchma & O Toole, 2022; Poulin & Kuperman, 2021; Ruhal & Kataria, 2021; Sırıken et al., 2021). Several key factors influencing this stage include surface sensing and adhesion, motility, physiological changes, reversible and irreversible attachment, and environmental influences.

A. Surface Sensing and Adhesion: Upon encountering a suitable surface, *P. aeruginosa* cells undergo a series of biochemical and physiological changes to initiate attachment (Jones et al., 2022). Surface sensing involves the recognition of physical and chemical properties, such as surface topography, hydrophobicity, and availability of binding sites (Lee et al., 2021). The bacterium utilizes specialized surface appendages and adhesins, such as type IV pili and adhesins like lectins and polysaccharides, to adhere to the surface (Guo et al., 2021; Kreve & Dos Reis, 2021; Whitfield & Brun, 2024).

B. Motility: *P. aeruginosa* known for its ability to use swimming, swarming, and twitching motilities (Ma et al., 2022). Swimming motility, mediated by flagella, helps planktonic cells reach and attach to surfaces (Zegad o et al., 2023). Once attached, twitching motility enables cells to crawl and aggregate, forming microcolonies (Carabelli et al., 2020). Twitching motility aids in exploring and colonizing surfaces (Ligthart et al., 2020), while swarming motility facilitates rapid surface coverage and expansion of biofilm communities (Worlitzer et al., 2022). These motilities contribute to the structural organization of biofilms, including initial attachment, microcolony formation, and the establishment of mature biofilm architecture (Guzm n-Soto et al., 2021).

Type IV pili, composed of pilin subunits, play a crucial role in initial attachment by mediating reversible adhesion to surfaces through retractile and twitching motility mechanisms (David et al., 2024; Neuhaus et al., 2020). These pili facilitate surface exploration and engagement, enhancing the likelihood of stable attachment (Mohamad et al., 2023).

C. Physiological Changes: During initial attachment, *P. aeruginosa* cells undergo physiological changes that optimize their adherence and prepare them for subsequent stages of biofilm development (Vetrivel et al., 2021). These changes may include alterations in gene expression patterns, metabolic shifts, and the synthesis of extracellular matrix components (Su et al., 2022). For instance, upregulation of genes involved in adhesion and initial colonization is often observed, ensuring robust attachment to the surface (P. Li et al., 2023).

D. Reversible and Irreversible Attachment: Initial attachment is typically reversible, allowing bacterial cells to explore multiple surfaces and optimize their positioning before committing to irreversible attachment and subsequent biofilm development (Sharma et al., 2023). This reversible attachment phase is crucial for biofilm initiation and enables *P. aeruginosa* to adapt to varying environmental conditions and surface properties (R. Yin et al., 2022).

E. Environmental Influences: Environmental factors such as nutrient availability, pH, temperature, and the presence of other microorganisms can influence the initial attachment process (X. Wang et al., 2023). *P. aeruginosa* has a remarkable ability to adapt to diverse environmental conditions, allowing it to colonize a wide range of surfaces and environments (Benigno et al., 2023).

Stage II: Microcolony Formation

After the initial attachment of planktonic cells to a surface, *P. aeruginosa* progresses to microcolony formation (Sarkar, 2020). This stage is characterized by the aggregation and clustering of attached bacterial cells into small, densely packed clusters known as microcolonies (Luo et al., 2022). Microcolony formation is a critical step in biofilm development, serving as a foundation for the subsequent stages of biofilm maturation and scaffolding (Alotaibi & Bukhari, 2021). Key factors influencing microcolony development include aggregation and QS, EPS production and biofilm matrix formation, and spatial organization and nutrient acquisition. These factors collectively impact the growth and structure of microcolonies, shaping the overall biofilm architecture.

A. Aggregation and Quorum Sensing

P. aeruginosa utilizes QS systems to coordinate the aggregation of cells into microcolonies (X. Liu et al., 2023). QS involves the production and sensing of signaling molecules (such as N-acyl homoserine lactones (AHLs) in the Las and Rhl systems), which accumulate as the bacterial population density increases (García-Reyes et al., 2020). QS systems regulate the expression of genes involved in cell-cell communication, EPS production, and biofilm maturation, all of which are crucial for microcolony formation (P. Li et al., 2023).

Table 1: *Pseudomonas aeruginosa* biofilm formation stages, including factors, structures, and conditions that facilitate each stage.

Stage	Factors and Structures	Conditions and Facilitators	References
Stage 1: Initial Attachment	- Surface Sensing and Adhesion	- Surface topography, hydrophobicity, availability of binding sites	(Schwibbert et al., 2024)
	- Motility and Adhesins (Type IV pili, twitching motility)	- Flagella-mediated swimming motility, twitching motility for surface exploration	(Cont et al., 2023; Pfeifer et al., 2022)
	- Physiological Changes	- Gene regulation (Quorum sensing systems: Las and Rhl), metabolic shifts, EPS production pathways	(Vetrivel et al., 2021)
	- Reversible and Irreversible Attachment	- Ability to explore surfaces before irreversible attachment	(Uneputty et al., 2022)
	- Environmental Influences	- Nutrient availability, pH, temperature, presence of other microorganisms	(Scribani Rossi et al., 2022)
Stage 2: Microcolony Formation	- Aggregation and Quorum Sensing	- QS signaling molecules (N-acyl homoserine lactones (AHLs)), cell density	(David et al., 2024)
	- EPS Production and Biofilm Matrix	- Polysaccharides (alginate, Pel), proteins, DNA; regulated by AlgZR, Pel systems	(Sultan et al., 2021) (R. Li et al., 2024)
	- Spatial Organization and Nutrient Acquisition	- Heterogeneous patterns, nutrient gradients, oxygen availability	(Pai et al., 2023)
Stage 3: Maturation and Stability	- EPS Production and Biofilm Architecture	- Alginate, Pel, Psl polysaccharides, extracellular DNA and proteins	(Le Mauff et al., 2022)(Singh et al., 2021)
	- Three-Dimensional Structure	- Channels, water channels for nutrient and waste exchange	(Uneputty et al., 2022)
	- Metabolic Activity and Phenotypic Heterogeneity	- Differential gene expression, metabolic adaptation	(Vohra et al., 2023)
Stage 4: Dispersion	- Triggering Factors	- Environmental cues (oxygen levels, nutrient depletion), signaling molecules	(Rumbaugh & Sauer, 2020)
	- Regulatory Mechanisms (Quorum Sensing)	- Las and Rhl systems, accumulation of QS signals	(Ambreetha & Singh, 2023)
	- Dispersal Strategies	- Enzymatic degradation (proteases, nucleases), surfactant production (rhamnolipids)	(Cherny & Sauer, 2020)(Carrazco-Palafox et al., 2021)
	- Physiological Changes	- Metabolic shifts for planktonic survival	(Thi et al., 2020)

B. Extracellular Polymeric Substances (EPS) Production and Biofilm Matrix
During microcolony formation, *P. aeruginosa* synthesizes and secretes EPS, which contribute to the structural integrity and matrix of the biofilm (Y. Li et al., 2021). EPS components include polysaccharides (e.g., alginate and Pel), proteins, and DNA, which together form a hydrated matrix that encases bacterial cells within the microcolony (Chung et al., 2023; Karygianni et al., 2020). EPS production is regulated by various genetic pathways, such as the AlgZR and Pel systems, which respond to environmental influence and QS signals to coordinate matrix synthesis and biofilm development (Sultan et al., 2021).

C. Spatial Organization and Nutrient Acquisition
Microcolonies exhibit spatial organization characterized by heterogeneous patterns of bacterial cells within the biofilm (Shree et al., 2023). This organization allows for nutrient gradients, oxygen gradients, and metabolic diversity, which support the growth and persistence of *P. aeruginosa* within the biofilm community (David et al., 2024; Tuon et al., 2022). Cells within microcolonies may display phenotypic heterogeneity, including differences in gene expression profiles and physiological states, contributing to biofilm resilience and adaptability (Ugolini et al., 2024).

Stage III: Maturation and Stability
As microcolonies mature, the biofilm architecture becomes more complex, with the formation of channels and water channels that facilitate nutrient and waste exchange (P. Li et al., 2023; Verma et al., 2023). Maturation involves continued EPS production, cell-cell interactions, and the recruitment of additional bacterial cells to the growing biofilm community (Sharma et al., 2023). This stage is essential for biofilm stability ,resistance to environmental stresses and antimicrobial agents (Grooters et al., 2024). Key factors influencing this stage include:

A. EPS Production and Biofilm Architecture
EPS production continues to be a critical aspect of biofilm maturation (Grooters et al., 2024). *P. aeruginosa* synthesizes and secretes EPS components such as alginate, Pel, and Psl polysaccharides, as well as extracellular DNA (eDNA) and proteins (Sarkar, 2020). EPS components contribute to the structural integrity of the biofilm matrix, forming a hydrated and protective environment that encases bacterial cells within the biofilm community (Bano et al., 2023; Gerardi et al., 2024). This matrix facilitates nutrient and waste exchange and protects against host immune responses and antimicrobial agents (Erkihun et al., 2024).

B. Three-Dimensional Structure
During maturation, *P. aeruginosa* biofilms develop a three-dimensional architecture characterized by complex arrangements of microcolonies, channels, and water channels (X. Wang et al., 2023). These structures allow for spatial organization, nutrient gradients, and oxygen availability within the biofilm community (Polizzi et al., 2022). Channels and water channels facilitate the flow of nutrients and metabolic waste products throughout the biofilm, supporting the growth and metabolic activity of bacterial cells within different regions of the biofilm (Quan et al., 2022).

C. Metabolic Activity and Phenotypic Heterogeneity
P. aeruginosa cells within mature biofilms exhibit metabolic adaptation and phenotypic heterogeneity. This includes variations in gene expression profiles, physiological states, and metabolic activities, which contribute to biofilm resilience and adaptability (Ugolini et al., 2024). Differential gene expression regulates metabolic pathways involved in energy production, nutrient acquisition, and stress responses, allowing *P. aeruginosa* to thrive in diverse environmental conditions encountered within the biofilm (Munir et al., 2020).

Stage IV: Dispersion
Dispersion is the process by which bacterial cells detach from the mature biofilm and return to a planktonic (free-floating) state. This stage is crucial for *P. aeruginosa* biofilms as it allows bacteria to disseminate, colonize new environments, and initiate new infections (Mancuso et al., 2024). Dispersion involves coordinated regulatory mechanisms that facilitate the release of bacterial cells from the biofilm matrix and their transition back to a planktonic lifestyle (Mukherjee et al., 2023).

A. Triggering Factors
Dispersion is triggered by environmental cues and signals that indicate changes in nutrient availability, stress conditions, or the need to colonize new surfaces or host tissues. These cues can include fluctuations in oxygen levels, nutrient depletion, or the presence of signaling molecules that regulate biofilm dispersal (Rumbaugh & Sauer, 2020).

B. Regulatory Mechanisms
P. aeruginosa utilizes QS systems, such as the Las and Rhl systems which regulate biofilm dispersion (Ju kov et al., 2023; D. Tang et al., 2024). QS signals accumulate within the biofilm as the bacterial population density increases, triggering the expression of genes involved in dispersion and planktonic growth (Juszczuk-Kubiak, 2024). QS systems control the production of dispersal factors, including enzymes and surfactants, that degrade the biofilm matrix and facilitate the release of bacterial cells (Borges et al., 2020; H. Wang et al., 2022).

C. Dispersal Strategies
1. Enzymatic Degradation: *P. aeruginosa* produces enzymes such as proteases and nucleases that degrade the EPS matrix and disrupts the structural integrity of the biofilm (Chaphalkar, 2023). This enzymatic activity promotes the dispersal of bacterial cells by breaking down the bonds that anchor cells to the biofilm matrix (Jiang et al., 2020).
2. Surfactant Production: Surfactants, such as rhamnolipids, are produced by *P. aeruginosa* during biofilm dispersion (Brindhadevi et al., 2020). These molecules reduce surface tension and facilitate the detachment of bacterial cells from the biofilm, promoting their release into the surrounding environment (Carrazco-Palafox et al., 2021).

D. Physiological Changes: Upon dispersal, *P. aeruginosa* cells undergo metabolic shifts to adapt to the planktonic lifestyle (Zemke et al., 2020). This includes changes in gene expression and metabolic pathways to support individual survival and growth in the new environment (David et al., 2024).

MOLECULAR REGULATION OF *Pseudomonas aeruginosa* BIOFILM FORMATION
P. aeruginosa biofilm formation is tightly regulated by a network of genes and signaling pathways that coordinate each stage of the biofilm lifecycle (S. Park & Sauer, 2021). These regulatory mechanisms ensure the adaptability, resilience, and pathogenic potential of *P. aeruginosa* biofilms in diverse environments and clinical contexts (Ugolini et al., 2024).

A. Initial Attachment:
The initial attachment phase in bacterial biofilm formation involves several key mechanisms. Type IV Pili and adhesins play essential roles, with PilA (Pilin) and PilB encoding the major pilin subunit and assembly proteins responsible for twitching motility and initial surface attachment (T. Zhang et al., 2023). Additionally, CdrA, an adhesin protein promotes adherence to both abiotic surfaces and host cells, facilitating initial attachment processes (Jacobs et al., 2022; Singh et al., 2021). Motility is governed by genes such as *Flh*(A-D) and *Fli*(D-T, Y, Z), which regulates the flagellar apparatus by encoding structural proteins like flagellin (*FliC*), motor proteins, and regulatory factors crucial for bacterial movement. *FlhDC* acts as a master regulator controlling the expression of these flagellar genes in response to environmental signals (G. Sun et al., 2023; H. Sun et al., 2022).
QS systems, including LasR-LasI and RhlR-RhlI, regulate the production of QS signal molecules such as N-acyl homoserine lactones (AHLs), influencing genes pivotal for initial attachment and early biofilm formation (Kanojiya et al., 2022; Sırıken et al., 2021). The *rhlAB* genes participate in rhamnolipid synthesis,

which reduces surface tension and facilitates swarming motility (Lavanya, 2024). Moreover, EPS production is governed by *Pel* and *Psl* biosynthesis genes, which synthesize extracellular polysaccharides (Pel and Psl) needed for biofilm matrix formation and enhancing initial surface attachment (Pezzoni et al., 2020; Soleymani-Fard et al., 2024). These mechanisms collectively contribute to the intricate process of bacterial biofilm initiation and development (Asma et al., 2022).

B. Microcolony Formation:

Microcolony formation during bacterial biofilm development involves intricate regulatory mechanisms. QS and biofilm formation genes, such as *LasR* and *RhlR* targets, play crucial roles in orchestrating this process (Pugazhendhi et al., 2022). They regulate the expression of genes essential for microcolony formation, including those responsible for EPS production (*algD*, *pelA*, and *pslA*), surface adhesion (*cupA1* and *cupA2*), and aggregation (*fimU*) (Hasan Kashkool & Al-Muhanna, 2020; Hern ndez-S nchez et al., 2024; Yam et al., 2022). Additionally, small RNA regulators like *RsmA* and *RsmZ* exert control over genes involved in biofilm formation. These include motility-related genes (*fleQ*) and EPS production genes (*pel* and *psl*), thereby influencing microcolony development and the overall structure of the biofilm (Condinho et al., 2023).

C. Maturation:

During the maturation phase of bacterial biofilm development, several key processes ensure stability, resilience, and adaptation (Y. Wang et al., 2024). The AlgZR system plays a pivotal role by regulating genes involved in alginate biosynthesis (*algD* and *algC*), which are crucial for the production of extracellular polysaccharides and the stabilization of the biofilm matrix (Pezzoni et al., 2022; Yam et al., 2022; Yeboah, 2021). Simultaneously, genes responsible for Pel and Psl synthesis remain actively expressed, reinforcing the biofilm matrix throughout maturation (Goel et al., 2021; Singh et al., 2021). This continuous synthesis contributes significantly to the architecture and resistance of the biofilm (Y. Li et al., 2020; Zhao et al., 2023).

Furthermore, the cyclic-di-GMP signaling pathway plays a central role in coordinating the transition between planktonic and biofilm lifestyles. This signaling system influences EPS production, modulates motility, and enhances stress responses as the biofilm matures (Luo et al., 2022). Together, these mechanisms ensure the robustness and adaptability of the biofilm structure during its maturation phase, allowing bacterial communities to persist and thrive in diverse environmental conditions (Diggle & Whiteley, 2020).

D. Dispersion:

Biofilm dispersion, the process by which bacteria transition from a sessile biofilm state to a planktonic lifestyle, involves orchestrated genetic regulation and environmental cues (Penesyan et al., 2021). The *LasR* and *RhlR* regulators play crucial roles in this phase by activating dispersal genes such as *rhlA* and *rhlB* (Sánchez-Jiménez et al., 2023). These genes encode enzymes and surfactants, notably rhamnolipids, which facilitate the breakdown of the biofilm structure and promote dispersal into the surrounding environment (Lavanya, 2024).

Additionally, environmental stress response genes are pivotal in triggering biofilm dispersal in response to various environmental cues, including nutrient depletion and fluctuating oxygen levels (P. Li et al., 2023). These genes enable bacteria to adapt and disperse from the biofilm in search of more favorable conditions (Luo et al., 2022). Together, these regulatory mechanisms and environmental responses ensure the dynamic balance between biofilm formation and dispersal, allowing bacteria to thrive and survive in diverse ecological niches (Abebe, 2020).

METHODOLOGIES FOR EVALUATING ANTIBIOFILM ACTIVITY

Evaluation of antibiofilm agents involves several crucial steps to assess their efficacy against bacterial biofilms. Initially, the antibacterial activity of potential agents is determined through methods such as broth microdilution to establish the minimum inhibitory concentration (MIC), indicating the lowest concentration that inhibits visible bacterial growth (Hossain, 2024). Subsequently, the evaluation shifts to examining antibiofilm activity at sub-MICs, focusing on different stages of biofilm development: initial attachment, microcolony formation, maturation, and dispersion (Erkihun et al., 2024). Techniques like crystal violet staining, confocal laser scanning microscopy, and viability assays are employed to quantify biofilm biomass and assess the impact of agents on biofilm integrity and viability at each stage (Diouchi et al., 2024). Effective antibiofilm agents not only inhibit biofilm formation but also disrupt existing biofilms, making them critical candidates for combating persistent bacterial infections (Kang et al., 2023).

A. Screening study

1. Crystal Violet Assay

The crystal violet assay is a widely employed method for quantifying biofilm biomass and evaluating the effectiveness of antibiofilm agents (Meneses *et al*., 2023). It operates on the principle of crystal violet dye binding to negatively charged components within the biofilm matrix, such as extracellular polysaccharides and proteins (Ramachandra et al., 2023). After incubation and subsequent washing to remove unbound dye, the bound dye is solubilized using solvents like ethanol or acetic acid, and its absorbance is measured spectrophotometrically at around 570 nm (Jha et al., 2023). This measurement correlates with the amount of biofilm biomass present, offering a semi-quantitative assessment of biofilm formation (Diouchi et al., 2024). The assay is valued for its simplicity, cost-effectiveness, and scalability, making it suitable for high-throughput screening in laboratory settings (Nilles et al., 2022). However, it does have limitations, including its lack of specificity in differentiating between live and dead cells within the biofilm and its inability to provide detailed structural information or spatial distribution of cells (Pisaruka, 2023). Researchers often complement the crystal violet assay with other techniques to gain a more comprehensive understanding of biofilm dynamics and the efficacy of antibiofilm treatments.

2. Cell Viability Assay

Cell viability assays are fundamental methods used to assess the number of viable (live) cells within a sample, crucial for evaluating the efficacy of antibiofilm agents (Di Bonaventura et al., 2023). It provides an indication about the antibiofilm activity rather than the bacteriostatic activity of the agent (Diouchi et al., 2024). These assays typically rely on markers of cell membrane integrity or metabolic activity to distinguish live cells from dead ones (Cai et al., 2024). For instance, fluorescent dyes like fluorescein diacetate (FDA) or calcein-AM penetrate live cells and produce fluorescence upon cleavage by intracellular esterases, indicating cell viability (Elisabeth et al., 2021). Alternatively, assays such as the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay measure metabolic activity through the reduction of tetrazolium salts by mitochondrial enzymes in viable cells (Elisabeth et al., 2021). The advantages of cell viability assays include their quantitative nature, allowing for precise comparisons between different treatments or experimental conditions (Larsson et al., 2020; Mikheeva et al., 2024). They are also highly sensitive, capable of detecting subtle changes in cell viability (Robinson et al., 2023). Moreover, these assays are adaptable to various cell types and can be applied to both planktonic and biofilm-associated cells (Dong et al., 2020). However, limitations include potential interference from dead cells that may retain some metabolic activity, as well as the complexity and cost associated with certain assay formats (Kamiloglu et al., 2020). Variability in experimental conditions such as incubation time and temperature can also impact assay results, requiring careful consideration in experimental design and interpretation (Larsson et al., 2020). Despite these challenges, cell viability assays remain indispensable tools in biomedical and microbiological research for assessing cellular health and response to treatments (Khalef et al., 2024).

Table 2: the key genes involved in *P. aeruginosa* biofilm formation stages, and their functions.

Stage	Gene(s)	Function	Reference(s)
Initial attachment	*PilA* (Pilin)	Major pilin subunit involved in type IV pili assembly	(Shanmugasundarasamy et al., 2022)
	PilB	Pilus assembly protein	(Dye & Yang, 2020)
	PilY1, *PilY2*	Adhesion and twitching motility	(Sultan et al., 2021)
	Flh(A-D)	Flagellar protein export apparatus	(Halte & Erhardt, 2021)
	FliE-FliR	Flagellar motor proteins and assembly factors	(Halte & Erhardt, 2021)
	FlhDC	Master regulator of flagellar gene expression	(Nedeljkovi et al., 2021)
	CdrA	Adhesin protein promoting adherence to surfaces	(Nedeljkovi et al., 2021; Singh et al., 2021)
	rhlAB	Rhamnolipid biosynthesis (surfactant production)	(Albasri et al., 2024)
	LasR-LasI RhlR-RhlI	QS system regulating AHL production and adhesion genes	(Bernabè et al., 2022)
	FleQ	Global regulator of flagellar and exopolysaccharide biosynthesis	(Dardis et al., 2021; Oladosu et al., 2024)
	Pel and *Psl*	Genes involved in EPS production and biofilm matrix formation	(Balducci et al., 2023; Feng et al., 2020; Grossich et al., 2023)
Microcolony Formation	*LasR* and *RhlR*	Regulate genes for EPS production (algD, pelA, pslA), adhesion (cupA1, cupA2), and aggregation (fimU)	(Elnegery et al., 2021)
	RsmA and *RsmZ*	Small RNAs regulating motility (fleQ) and EPS production (pel, psl)	(Sánchez-Jiménez et al., 2023)
Maturation	*AlgZR* System	Regulates alginate biosynthesis genes (algD, algC)	(Pezzoni et al., 2022; Yeboah, 2021)
	Pel and *Psl*	Continuously expressed genes for EPS synthesis and biofilm matrix reinforcement	(M. Tang et al., 2024)
	Cyclic-di-GMP Signaling	Controls EPS production, motility, and stress responses	(Krol et al., 2020)
Dispersion	*LasR* and *RhlR* Regulation	Activates dispersal genes (e.g., rhlA, rhlB) for enzymes and surfactants (e.g., rhamnolipids)	(Castro et al., 2022)

Table 3: Microscopy techniques used for biofilm visualization, along with their advantages and disadvantages:

Microscopy Technique	Advantages	Disadvantages	References
Brightfield Microscopy	- Simple and widely available. - Provides general observation of biofilm morphology. - Shows the general aggregation of cells within a biofilm. - Requires minimal sample preparation.	- Limited contrast, especially for transparent biofilm components like EPS. - Cannot differentiate live vs. dead cells without staining	(Futo et al., 2022; Kozlova et al., 2020; Y. Liu et al., 2022)
Phase-Contrast Microscopy	- Enhances contrast of transparent structures (e.g., EPS) without staining. - It can give a 2D representation of the biofilm structure, showing surface features and some spatial organization - Allows for visualization of live biofilm samples.	- Limited to contrast enhancement and does not provide detailed structural information.	(Yuan et al., 2020)
Differential Interference Contrast (DIC) Microscopy	- Provides 3D-like images with enhanced depth perception. - High resolution and detailed visualization of biofilm architecture.	- Requires skilled operation and calibration. - Can be costly to implement and maintain.	(Han et al., 2023)
Fluorescence Microscopy	- Enables specific labeling and visualization of biofilm components using fluorescent dyes or proteins. - Allows for quantitative analysis of biomolecules.	- Requires fluorescent labeling, which can alter biofilm dynamics. - High cost of fluorescent probes and equipment.	(Guéneau et al., 2023; Panda et al., 2021)
Confocal Laser Scanning Microscopy (CLSM)	- Provides high-resolution, 3D imaging of biofilms. - Allows for real-time observation and dynamic imaging. - Penetrates thick biofilms for detailed analysis.	- Expensive equipment and maintenance. - Requires expertise in operation and data analysis. - Depth limitations in thick biofilms.	(Albalawi, 2024; Elliott, 2020; Mhade & Kaushik, 2023)
Scanning Electron Microscopy (SEM)	- Provides high-resolution images (down to nanometer scale), revealing detailed surface structures and topography of biofilms. - Facilitates elemental analysis using backscattered electrons (BSE)	- Requires meticulous sample preparation, including dehydration and coating. - High initial cost of equipment and ongoing maintenance. - Complex operation and interpretation, necessitating trained personnel. - Limited depth of field, making it challenging to image entire biofilm thicknesses in focus without specialized techniques.	(Y. Huang et al., 2020; Silva et al., 2021)

3. Microscopic visualization of biofilm

Visualizing biofilms using microscopy techniques is essential for gaining detailed insights into their structural organization, composition, and dynamics (Table 3). Optical microscopy methods such as brightfield, phase-contrast, and differential interference contrast (DIC) microscopy operate on the principle of visible light absorption, refraction, and interference, respectively, to visualize biofilms (Dubay et al., 2023). Brightfield microscopy provides contrast between biofilm components based on light absorption by cells and extracellular matrix (ECM), offering a straightforward observation method (Clapperton et al., 2024). Phase-contrast microscopy enhances contrast by detecting differences in refractive indices within the biofilm, making transparent structures like EPS more visible without the need for staining (Albalawi, 2024). DIC microscopy further improves visualization by detecting differences in optical path length, producing 3D-like images that highlight biofilm architecture and cell arrangements (Han et al., 2023).

Fluorescence microscopy extends visualization capabilities by using fluorescent dyes or genetically encoded fluorescent proteins (e.g., Green Fluorescent Protein) to selectively label biofilm components (Hickey et al., 2021). This technique allows researchers to track specific biomolecules, such as cells or extracellular matrix materials, within the biofilm matrix (Blanco-Romero et al., 2024). Confocal laser scanning microscopy (CLSM), a powerful tool in biofilm research, combines fluorescence microscopy with laser scanning technology to generate high-resolution, three-dimensional images of biofilms (Gerardi et al., 2024). CLSM can penetrate thick biofilms and provide detailed spatial information on biofilm structure, cell distribution, and viability in real-time (Waqas et al., 2023).

Scanning Electron Microscopy (SEM) is a sophisticated imaging technique widely utilized in biofilm research for its ability to provide detailed, high-resolution images of surface structures (A. Ali et al., 2023). SEM imaging reveals topographical details of biofilm surfaces, offering insights into the arrangement of cells, EPS, and other surface features (Cleaver & Garnett, 2023). Backscattered Electrons imaging, on the other hand, distinguishes materials based on their atomic number, facilitating elemental analysis within biofilms (A. Ali et al., 2023). SEM's high magnification capabilities (up to 100,000x or more) enable researchers to examine biofilm ultrastructure with exceptional clarity, though it requires careful sample preparation involving dehydration and coating with a conductive layer to optimize imaging conditions (Gaines et al., 2024; A. J. Park et al., 2021). Despite its complexity, cost, and technical demands, SEM remains indispensable for advancing our understanding of biofilm morphology, surface interactions, and composition at the microscopic scale (Cleaver & Garnett, 2023).

The advantages of microscopy in biofilm visualization include high spatial resolution, enabling detailed examination of biofilm architecture and cellular interactions (Cleaver & Garnett, 2023; Relucenti et al., 2021). Real-time imaging capabilities of CLSM facilitate dynamic observations of biofilm growth, development, and responses to environmental changes or antimicrobial treatments (Mhade & Kaushik, 2023). Fluorescence microscopy offers flexibility in labeling and quantitative analysis of biofilm

components, allowing for precise measurements of biofilm biomass, thickness, and cellular density (Idrees et al., 2021). However, these techniques have limitations, such as sample preparation requirements that can alter biofilm structure and introduce artifacts (Relucenti et al., 2021). Depth limitations in optical microscopy techniques may restrict imaging of biofilm layers deeper within the matrix, while advanced microscopy methods like CLSM can be expensive and require specialized expertise for operation and data interpretation (Albalawi, 2024). Despite these challenges, microscopy remains indispensable for advancing our understanding of biofilm biology and developing strategies to combat biofilm-associated infections (Cleaver & Garnett, 2023).

B. Target identification

1. Surface charge, aggregation, and hydrophobicity

Surface charge and hydrophobicity are pivotal factors influencing the initial attachment of microorganisms during biofilm formation (Zhao et al., 2023). Surface charge refers to the electrical charge of a material's surface, which arises from the presence of functional groups capable of ionization, such as carboxyl or amino groups (Perchikov et al., 2024). This charge influences the interaction between the material and microbial cells through electrostatic forces. Techniques like zeta potential analysis and electrophoretic mobility are used to quantify surface charge. A high zeta potential indicates strong repulsive forces between similarly charged particles, potentially preventing microbial attachment. Conversely, oppositely charged surfaces can attract microbial cells, facilitating adhesion and subsequent biofilm formation (Ditu et al., 2024). Factors such as pH and ionic strength of the surrounding medium significantly influence surface charge by affecting the ionization state of functional groups on the surface (K. Wang et al., 2024).

Hydrophobicity, on the other hand, refers to the tendency of a surface to repel water molecules (Crago et al., 2024). This property plays a critical role in microbial attachment as many microorganisms are inherently hydrophobic and preferentially adhere to hydrophobic surfaces (Elfazazi et al., 2021). Measurement techniques like contact angle measurement and microbial adhesion to hydrocarbons are employed to assess surface hydrophobicity (Schneier et al., 2024). A higher contact angle indicates greater hydrophobicity, correlating with increased microbial adhesion (Bohinc et al., 2024; Fouda et al., 2024; Nakanishi et al., 2021). Hydrophobic interactions between microbial cells and surfaces contribute to the stability and cohesion of biofilms, as these interactions enhance the initial attachment and subsequent growth of biofilm communities (Afrasiabi & Partoazar, 2024). Surface roughness and composition are key factors influencing surface hydrophobicity, with rougher surfaces and those containing hydrophobic functional groups exhibiting higher hydrophobicity (Macko et al., 2022).

In biofilm formation, understanding surface charge and hydrophobicity is crucial for designing strategies to control microbial attachment (Zhao et al., 2023). By manipulating these surface properties, researchers can develop materials and coatings that either promote or inhibit biofilm formation, depending on the desired application (W. Yin et al., 2021). This knowledge is particularly relevant in healthcare settings, industrial processes, and environmental management, where biofilm formation can impact equipment performance, product quality, and ecosystem health (Almatroudi, 2024). Thus, detailed characterization and manipulation of surface charge and hydrophobicity provide valuable insights into the mechanisms of initial microbial attachment and biofilm development, enabling targeted interventions to manage biofilm-related issues effectively (Shi et al., 2022).

2. Bacterial Motility

Evaluation of microbial motility, encompassing behaviors such as swarming, swimming, and twitching, involves tailored techniques for each type of movement (Palma et al., 2022). Swimming motility, where bacteria navigate through liquid environments using flagella or other appendages, is commonly assessed through soft agar assays. In these assays, bacteria are inoculated into semi-solid nutrient agar plates with lower agar concentrations (typically 0.3-0.5%), allowing them to move freely (Partridge & Harshey, 2020). Over time, the spread of bacteria away from the point of inoculation indicates their swimming ability (Gude et al., 2020). Microscopy, particularly phase-contrast or dark-field microscopy, is also employed to observe and quantify swimming speed and directionality of individual cells, providing detailed insights into their motility characteristics (Bente et al., 2020; Palma et al., 2022; Z. Zhang et al., 2023).

Swarming motility, characterized by collective movement of bacteria over solid surfaces aided by flagella and EPS, is evaluated using swarming plates (Bru et al., 2023). These plates have nutrient agar with slightly lower agar concentrations (0.5-0.7%), which permits bacterial swarming (Hausmann et al., 2021). Observing the colony expansion rate (swarming diameter) and patterns such as dendritic or branching growth provides qualitative and quantitative data on swarming behavior (Guo et al., 2022; Priyadarshini, 2024). Techniques like time-lapse microscopy and image analysis further enable tracking and measuring parameters such as colony area, expansion velocity, and morphology, offering deeper insights into the dynamics of swarming motility (Partridge et al., 2023).

Twitching motility, involving surface-associated movement facilitated by extension and retraction of type IV pili or similar appendages, is typically assessed through agar plate assays (Barton et al., 2024). In these assays, bacteria are stab-inoculated into nutrient agar plates and allowed to incubate for extended periods (24-72 hours) (Rayamajhee et al., 2024). The formation of thin, spreading growth zones (halos) around the point of inoculation indicates twitching motility. Microscopy is also employed to visualize bacterial cells at the edge of these zones, providing direct observation of pili-mediated movement, including the cycles of extension and retraction (Kühn et al., 2021; Thongchol et al., 2024).

3. Extracellular Polymeric Substance (EPS) production

Studying the effect of antibiofilm agents on EPS production involves a range of methods aimed at understanding how these agents influence the matrix that holds microbial biofilms together. EPS is crucial for biofilm formation and stability, serving as a protective barrier against antimicrobial agents and environmental stresses (Kadirvelu et al., 2024).

One of the primary approaches involves extracting EPS from biofilm samples treated with and without the antibiofilm agent. Methods for extraction include sonication, enzymatic digestion, or chemical extraction, depending on the nature of the EPS components (e.g., polysaccharides, proteins) (L. Huang et al., 2022). Quantification typically involves colorimetric assays such as the Bradford assay for proteins and the phenol-sulfuric acid method for polysaccharides (Bublitz et al., 2024; Reinmuth-Selzle et al., 2022). By comparing the total amount of EPS extracted from treated and untreated samples, researchers can assess the impact of the antibiofilm agent on EPS production (Burlacchini et al., 2024).

CLSM allows for the visualization and quantification of EPS within biofilms (X. Huang et al., 2022). By staining EPS components with fluorescent dyes specific to polysaccharides or proteins, researchers can observe spatial distribution and relative abundance of EPS in response to the antibiofilm agent (Lu et al., 2023). Image analysis software aids in quantifying fluorescence intensity or volume of EPS, providing insights into how the agent affects the structure and composition of the biofilm matrix (Pinto et al., 2020).

Enzymatic degradation assays assess the susceptibility of EPS to enzymatic degradation, which can be indicative of changes induced by antibiofilm agents (Thorn et al., 2021). Biofilm samples are treated with enzymes known to degrade EPS components (e.g., DNase for DNA, proteinase K for proteins) (Jiang et al., 2020; Karygianni et al., 2020). Reduction in EPS content after enzymatic treatment indicates the presence and composition of EPS in the biofilm matrix, and changes in degradation patterns can highlight alterations caused by the antibiofilm agent (Amankwah et al., 2021).

FTIR spectroscopy provides insights into the chemical composition of EPS (Gupta et al., 2021). By analyzing FTIR spectra of biofilm samples treated with and without the

antibiofilm agent, researchers can identify characteristic peaks corresponding to EPS components such as polysaccharides and proteins (Kowsalya et al., 2023). Changes in peak intensities or ratios between treated and untreated samples indicate modifications in EPS composition induced by the antibiofilm agent, offering qualitative and semi-quantitative data on molecular alterations within the biofilm matrix (Mirghani et al., 2022).

4. Rhamnolipids production

Studying the effect of antibiofilm agents on rhamnolipid production involves employing a variety of specialized methods aimed at quantifying and understanding changes in the synthesis of these important biosurfactants by bacteria, particularly *P. aeruginosa* (Sarubbo et al., 2022). Rhamnolipids play a significant role in biofilm formation and maintenance, contributing to the structural integrity and surface colonization abilities of microbial communities (Ma tkov et al., 2021).

One fundamental approach is the extraction and quantification of rhamnolipids from bacterial cultures treated with and without the antibiofilm agent (Firdose et al., 2021). This process typically involves using organic solvents such as chloroform or methanol to extract rhamnolipids from bacterial biomass (Bapat et al., 2022; Buhori et al., 2024). Quantification can be achieved using techniques like spectrophotometer, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), or mass spectrometry (MS) (El-Housseiny et al., 2020; Zompra et al., 2022). These methods allow researchers to measure the concentration and composition of rhamnolipids, providing insights into how the antibiofilm agent affects their production (Twigg et al., 2021).

Another crucial method is assessing the surface activity of rhamnolipids produced by bacteria (Albasri et al., 2024; Safari et al., 2023). Surface tension reduction assays, such as the drop-collapse method or oil-spreading assay, are commonly employed to evaluate the ability of rhamnolipids to reduce surface tension or emulsify hydrophobic compounds (Adetunji & Olaniran, 2021; Ghazi Faisal et al., 2023; Samuel-Osamoka et al., 2023). By comparing the surface activity of rhamnolipids from treated and untreated cultures, researchers can gauge changes in rhamnolipid production induced by the antibiofilm agent (S. Yang et al., 2023).

Liquid Chromatography-Mass Spectrometry (LC-MS) is a powerful tool for analyzing the composition and structure of rhamnolipids (Eslami et al., 2020). This technique allows for the separation, detection, and quantification of rhamnolipid molecules based on their mass-to-charge ratio (m/z) (Fu et al., 2020). By comparing LC-MS profiles of rhamnolipids from treated and untreated cultures, researchers can identify qualitative and quantitative changes in rhamnolipid production induced by the antibiofilm agent (Hijazi et al., 2023; Padaga et al., 2024).

5. Acyl homoserine lactone (AHL) production

Studying the impact of antibiofilm agents on acyl homoserine lactone (AHL) production in bacteria involves employing specialized methods to quantify and analyze changes in these signaling molecules crucial for QS. AHLs regulate various bacterial behaviors, including biofilm formation and virulence factor expression (Vashistha et al., 2023).

One fundamental approach is extracting AHLs from bacterial cultures treated with and without the antibiofilm agent using organic solvents like ethyl acetate or dichloromethane (Stock et al., 2021). AHL quantification can be achieved through bioassays with AHL-responsive biosensor strains such as *Chromobacterium violaceum* or analytical techniques such as LC-MS or Gas Chromatography-Mass Spectrometry (GC-MS) (Hor ek et al., 2023). These methods provide quantitative data on AHL concentration and profile, revealing how the antibiofilm agent affects their production (Stock et al., 2021).

6. Genes expression

The genes in *P. aeruginosa* associated with biofilm formation and regulation, presented in Table 4.

A. Genes Involved in Biofilm Matrix Biosynthesis: *P. aeruginosa* utilizes several genes to synthesize key components of its biofilm matrix (Blanco-Romero et al., 2024). The *pel* gene cluster is crucial for Pel polysaccharide biosynthesis, a major constituent that promotes biofilm structural integrity and adherence to surfaces (Balducci et al., 2023). Similarly, the *psl* operon contributes to the biosynthesis of Psl polysaccharide, which enhances biofilm stability and protects against environmental stresses (Chung et al., 2023). Additionally, the *alg* genes are involved in alginate biosynthesis, particularly important in mucoid strains where alginate forms a hydrated matrix, aiding in biofilm formation and protection (Char za et al., 2023).

B. Adhesion and Attachment Genes: Surface attachment is facilitated by genes encoding type IV pili (*pil*) and fimbrial adhesins (*fim*) (Aleksandrowicz et al., 2021). Type IV pili play a crucial role in initial attachment to surfaces and mediate twitching motility, essential for biofilm initiation (Ahmad et al., 2023; Odermatt et al., 2023). Fimbrial adhesins further contribute to the adherence of *P. aeruginosa* cells to both biotic and abiotic surfaces, initiating biofilm formation and colonization (Reichhardt, 2023).

C. Quorum Sensing and Regulatory Genes: QS systems in *P. aeruginosa*, notably the Las and Rhl systems, regulate biofilm formation and virulence gene expression (Shao et al., 2020; D. Yang et al., 2021). The *las* genes, including *lasI* (encoding AHL synthase) and *lasR* (encoding AHL receptor), control the Las system, which coordinates early biofilm formation (de Oliveira Pereira et al., 2023). Similarly, the *rhl* genes, such as *rhlI* and *rhlR*, regulate the Rhl system, influencing biofilm maturation and virulence factor production (Asfahl et al., 2022). Regulatory genes like *gacA/gacS* and *vfr* also play pivotal roles in coordinating biofilm formation through global regulatory networks, impacting gene expression and biofilm development (Coggan et al., 2022; Dela Ahator et al., 2022; Song et al., 2023).

D. c-di-GMP Signaling Pathway Genes: The cyclic diguanylate monophosphate (c-di-GMP) signaling pathway controls the switch between planktonic and biofilm lifestyles in *P. aeruginosa* (Kennelly et al., 2024). Genes encoding diguanylate cyclases (DGCs) and phosphodiesterases (PDEs) modulate intracellular levels of c-di-GMP, influencing biofilm initiation, maturation, and dispersal (Banerjee et al., 2021). Elevated c-di-GMP levels promote biofilm formation by enhancing EPS production and reducing motility, whereas lower levels favor planktonic growth and dispersal from the biofilm (S. Park & Sauer, 2022).

CHALLENGES

Understanding and evaluating *P. aeruginosa* biofilm formation present several challenges that impact both research and clinical applications. A significant challenge lies in the standardization of experimental methods across studies. There is considerable variability in growth conditions, media formulations, and biofilm quantification techniques used in different research settings (Coenye et al., 2024). This lack of consistency makes it difficult to compare results between studies accurately and limits the reproducibility of findings. Additionally, the complex three-dimensional structure of *P. aeruginosa* biofilms poses challenges in assessing biomass, architecture, and mechanical properties using conventional analytical methods (H. R. Ali et al., 2024). The heterogeneous nature of biofilms, with layers of cells embedded in an extracellular matrix, requires advanced imaging and characterization techniques to capture their full complexity (Cometta et al., 2024).

Biofilm-associated antimicrobial resistance is another critical challenge (Pai et al., 2023). *P. aeruginosa* biofilms exhibit inherent resistance mechanisms, making them difficult to eradicate with conventional antibiotics and immune responses (Omran et al., 2024). Understanding the mechanisms underlying biofilm resistance is crucial for developing effective treatment strategies (Grooters et al., 2024). Moreover, biofilms are

dynamic structures that undergo changes in response to environmental cues and stressors (X. Wang et al., 2023). Studying the temporal dynamics of biofilm formation, dispersion, and adaptation requires sophisticated experimental approaches that can monitor these processes in real-time.

FUTURE PERSPECTIVES

Moving forward, several future perspectives can enhance the study and management of *P. aeruginosa* biofilms. Standardization of experimental protocols and guidelines is paramount to improve the reliability and comparability of biofilm research outcomes. Establishing consensus on growth conditions, biofilm assays, and data interpretation will facilitate more robust and reproducible research across laboratories.

Advancements in imaging technologies and analytical methods offer promising avenues for studying biofilm structure and dynamics at higher resolutions. Techniques such as confocal laser scanning microscopy, cryo-electron microscopy, and omics approaches can provide detailed insights into biofilm composition, gene expression profiles, and metabolic activities. These tools enable researchers to better understand the underlying mechanisms driving biofilm formation and resistance.

Targeting biofilm-specific mechanisms represents another critical area of focus. Future research should explore novel therapeutic strategies that disrupt key pathways involved in *P. aeruginosa* biofilm formation, such as QS, exopolysaccharide production, and persister cell formation. By targeting these specific mechanisms, researchers aim to develop more effective treatments that can penetrate and eradicate biofilms effectively.

Collaborative efforts across multidisciplinary fields—including microbiology, bioinformatics, engineering, and clinical sciences—are essential for advancing biofilm research. Integrating diverse expertise can foster innovation in biofilm prevention, detection, and treatment strategies. Moreover, emphasizing translational research to validate promising antibiofilm agents and therapies in clinical settings is crucial. Conducting well-designed clinical trials will be instrumental in demonstrating the efficacy, safety, and feasibility of new biofilm treatments and ultimately improving patient outcomes.

CONCLUSION

In conclusion, understanding the biofilm formation process of *P. aeruginosa* is crucial for addressing the challenges posed by its chronic infections. This review highlights the importance of the various developmental stages of biofilms, presenting conventional screening methods that are fundamental for biofilm evaluation. Furthermore, we emphasize the intricate roles of genes and QS in regulating biofilm architecture and function. This comprehensive examination sheds light on the complex interactions involved in biofilm development, which are critical for devising innovative approaches to prevent and treat *P. aeruginosa* biofilm-associated infections. Future research should focus on integrating these insights into therapeutic strategies aimed at disrupting biofilm formation and enhancing treatment efficacy.

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Table 4: The most common genes involved in *Pseudomonas aeruginosa* biofilm formation and regulation

Category	Genes	Function	References
Biofilm Matrix Biosynthesis	Pel pellicle loci	Pel polysaccharide biosynthesis	(Whitfield et al., 2020)
	Psl Polysaccharide synthesis locus	Psl polysaccharide biosynthesis	(Chung et al., 2023)
	Alg	Alginate biosynthesis (in mucoid strains)	(J. Wang et al., 2023)
Adhesion and Attachment	Pil	Type IV pili involved in surface attachment and twitching motility	(Chen et al., 2022)
	Fim	Fimbrial adhesins facilitating initial surface adherence	(Govindarajan & Kandaswamy, 2022)
Quorum Sensing and Regulation	lasI, lasR	Las system: AHL synthesis and receptor	(Schuster et al., 2023)
	rhlI, rhlR	Rhl system: AHL synthesis and receptor	(Groleau et al., 2020)
	gacA/gacS	Gac/Rsm pathway regulatory genes controlling biofilm and virulence	(Song et al., 2023)
	Vfr	Global regulator influencing virulence and biofilm formation	(Coggan et al., 2022; Dela Ahator et al., 2022)
c-di-GMP Signaling	DGCs	Diguanylate cyclases regulating c-di-GMP synthesis	(Bhasme et al., 2020)
	PDEs	Phosphodiesterases controlling c-di-GMP degradation and turnover	(Bhasme et al., 2020; Feng et al., 2020)

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