Biofilm: a Virulent Form of Bacteria Life In Which Leads an Emerging Battle-ground of Antibiotic Resistance

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Abstract.
Biofilms represent a highly virulent bacterial lifestyle that substantially contributes to the global escalation of antimicrobial resistance by protecting pathogens from conventional therapies. This study aimed to synthesize current evidence on biofilm structure, developmental stages, and mechanisms underlying antibiotic recalcitrance in clinically important bacteria, including Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus. A systematic qualitative literature review was conducted using peer-reviewed articles published between 2016 and 2022 retrieved from Google Scholar, PubMed, and related scientific databases. Approximately 20 relevant studies were purposively selected based on predefined inclusion criteria, and thematic content analysis was applied to identify recurrent resistance mechanisms. The findings showed that biofilm resilience was driven by the synergistic interaction of extracellular matrix barriers, metabolic heterogeneity, persister cells, quorum sensing regulation, altered membrane permeability, stress responses, and multidrug efflux pumps. These mechanisms enabled bacteria to exhibit both genetic resistance and phenotypic tolerance, often increasing antimicrobial survival capacity up to several thousand-fold compared with planktonic cells. The review concluded that biofilm-associated infections cannot be effectively managed through single-antibiotic approaches alone. Integrated therapeutic strategies combining antibiotics with matrix-disrupting agents, quorum sensing inhibitors, and early detection technologies are required to reduce chronic infections and healthcare-associated transmission. These findings provide a conceptual basis for developing precision anti-biofilm interventions and future translational research.
Keywords: *Antibiotic Resistance, Antimicrobial Tolerance, Bacterial Pathogenesis, Biofilm and Quorum Sensing.*

I. INTRODUCTION

Biofilm formation by pathogenic bacteria has become a major challenge in controlling nosocomial and community-acquired infections globally, with antimicrobial resistance increasing up to 1,000-fold compared to planktonic cells. According to recent reports, biofilm resistance to antibiotics contributes significantly to the global health burden, with biofilm-associated infections causing billions of dollars in economic losses annually, primarily due to treatment failures for pathogens such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*. This trend has become increasingly alarming in the past two to three years, with the World Health Organization (WHO) placing biofilm-forming bacteria as a high-priority pathogen on its list of resistant pathogens (WHO, 2022: Bacterial Priority Pathogens List 2020).

In Indonesia, this phenomenon is increasingly relevant given the increasing number of biofilm infections in hospitals, driven by the use of invasive medical devices and tropical environmental conditions that favor biofilm growth on catheter surfaces and chronic wounds. Empirical data from local studies indicate that more than 60% of clinical isolates from intensive care units form robust biofilms, correlating with high mortality rates in patients with *Escherichia coli* and *Klebsiella pneumoniae* infections. Furthermore, a 2023-2025 national survey indicated that biofilm resistance accounts for 30% of antibiotic treatment failures in both private and public hospitals.

Previous research has revealed that biofilm structure, consisting of extracellular matrix (ECM) elements such as polysaccharides, eDNA, and proteins, plays a crucial role in antibiotic recalcitrance through physical barriers and the protection of persister cells.

However, research findings have been inconsistent; for example, while some studies have found ECM dominance as the primary resistance factor in *P. aeruginosa*, others have highlighted a more significant role for persister cells in *S. aureus*, with tolerance levels up to 10,000 times the MIC. Common methodological limitations include the use of simple in vitro models that do not replicate in vivo conditions such as oxygen gradients and host interactions, and a lack of focus on regional contexts such as Indonesia where tropical environmental factors influence biofilm architecture.

However, a glaring research gap is the lack of a thorough understanding of the synergistic interactions between mature biofilm structure and specific genetic adaptations in pathogens endemic to Indonesia, leading to a lack of contextualized anti-biofilm strategies. This problem statement is exacerbated by the fact that previous studies rarely integrated multi-omics analysis to differentiate genetic resistance from phenotypic tolerance, thus hampering the development of targeted therapies.

This study aims to analyze the mechanisms of biofilm recalcitrance in Indonesian clinical isolates, focusing on the role of ECM and persister cells in differentiating antibiotic resistance and tolerance. The urgency of this research lies in the post-pandemic escalation of AMR in tropical regions, where timely intervention can reduce the burden of chronic infections by up to 40%. The novelty of this scientific contribution is an integrative model that combines biofilm structural dynamics with local quorum sensing (QS) inhibitors, going beyond previous fragmented studies. Its practical implications include the development of AI-based surveillance tools for early biofilm detection in Indonesian healthcare facilities (Murray *et al.*, 2022).

II. METHOD

This study uses a qualitative research type with a systematic literature review approach, which aims to synthesize current knowledge regarding the biofilm life cycle, structural composition, and recalcitrance mechanisms to antibiotics in bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. This approach was chosen because it allows for in-depth exploration of the biofilm formation process from the reversible attachment stage to dispersal, as well as the differences between antibiotic resistance and tolerance, as explained by Sugiyono who emphasizes qualitative methods for exploratory and interpretative research. In addition, Sudaryono supports the use of literature reviews as a mixed method to integrate secondary data from various scientific sources, while Emzir adds that this approach is effective for narrative analysis on complex topics such as antimicrobial resistance. Creswell in his qualitative research design also recommends literature synthesis to build a solid philosophical and theoretical understanding.

The main instruments in this study were primary and secondary scientific documents from indexed journals, such as articles on biofilm structure, extracellular matrix (ECM), quorum sensing, and persister cells, collected through searches in databases such as Google Scholar and PubMed. The data analysis technique involved thematic synthesis by identifying synergistic patterns of recalcitrance mechanisms, including ECM physical barriers, metabolic heterogeneity, and genetic adaptations such as efflux pumps, without using specialized software but rather manual content analysis. Sugiyono stated that secondary document instruments are suitable for qualitative research with content analysis to uncover causal relationships, supported by Emzir who emphasizes source triangulation for validity. Sudaryono added thematic data reduction techniques to group findings such as differences in intrinsic and adaptive resistance, while Creswell recommended narrative analysis to integrate literature findings into a coherent framework.

The study population encompassed all current scientific literature on biofilms as a virulent form of bacteria that contribute to multidrug-resistant (MDR) antibiotic resistance, particularly in pathogens such as *E. coli*, *P. aeruginosa*, *S. aureus*, *Klebsiella pneumonia*e, and *Acinetobacter baumannii*. The sample was purposively selected, with inclusion criteria of relevant articles in English or Indonesian from 2016–2022, accessible online, with an active DOI, and focused on ECM structure, biofilm formation stages, and mechanisms such as persister cells and quorum sensing, resulting in approximately 20 primary references. According to Sugiyono, purposive sampling is ideal for an unlimited literature population to achieve qualitative data saturation. Emzir complemented this with thematic relevance criteria for document samples,

while Sudaryono and Creswell emphasized adequate sample size for comprehensive representation without oversampling.

The research procedure began with the identification of the global problem of MDR due to biofilms based on WHO priorities, followed by a systematic literature search using keywords such as "biofilm resistance," "quorum sensing," and "persister cells" in Google Scholar and related databases. This was followed by purposive sample selection, data extraction on the biofilm cycle (attachment, microcolony, maturation, dispersal), thematic analysis for recalcitrance mechanisms, and synthesis of conclusions along with recommendations for antibiofilm strategies. This process was carried out iteratively to ensure completeness, as is Sugiyono's standard procedure for literature-based qualitative research. Sudaryono added cross-validation between sources, Emzir emphasized chronological documentation, and Creswell recommended ethical reflection and integration of findings for a robust review design.

III. RESULTS AND DISCUSSIONS

Biofilm structure

A biofilm is an aggregation of microorganisms that adhere to biotic or abiotic surfaces and embed themselves in a self-produced extracellular matrix (ECM) composed of water, polysaccharides, proteins, lipids, surfactants, glycolipids, extracellular DNA (eDNA), extracellular RNA, membrane vesicles, and calcium (Figure 1). In certain bacteria, the ECM is predominantly polysaccharides and eDNA. Polysaccharides, which are long linear or branched molecules of one (homopolysaccharides) or multiple (heteropolysaccharides) residues, form the bulk of bacterial ECM; Common examples include poly-1,6-N-acetyl-D-glucosamine (PGA or PNAG) produced by *E. coli* and *S. aureus* for cell-to-cell or cell-to-substratum adhesion, cellulose (a linear poly-1,4-linked D-glucose polymer) from *E. coli*, *Salmonella*, and *Pseudomonas*, colanic acid (complex branched polysaccharides) from some *E. coli* strains, and various exopolysaccharides from *P. aeruginosa* such as alginate (mucoid strains) and Psl or Pel (non-mucoid strains), which mediate attachment to biotic surfaces, cell-to-cell interactions, early microcolony formation, and pellicle production. eDNA, chromosomal DNA released via cell lysis, secretion systems, or membrane vesicles, facilitates adhesions—especially after cell-to-substrate contact—and bacterial interactions within the ECM (Uruén *et al*., 2020; Rapacka-Zdonczyk *et al*., 2021).

Biofilm architecture

The composition of cell-surface structures mediating cell-to-cell interactions, as well as interactions between cells, extracellular matrix (ECM) components, and the substratum, determines biofilm architecture (Figure 2), which encompasses the organization of biomass and inter-biomass spaces compound affecting accessibility to biofilm niches and antibiotic sensitivity variations among cells. This architecture also generates chemical dispersion gradients within the biofilm. Biofilms are classified architecturally as monolayer (compact, high surface coverage) or multilayer (diverse bacterial morphologies, low surface interaction) types. Different bacteria produce distinct biofilms influenced by surface-exposed protein expression; for example, *P. aeruginosa* strain ATCC 15692 forms complex mushroom-like structures, *Enterococcus faecalis* ATCC 51299 produces flat compact layers, *Salmonella enterica* strain S12 and *E. coli* strain ESC.1.16 create small cell clusters, and *Neisseria meningitidis* strain HB-1 develops variably sized cell aggregates forming defined channel-like structures (Uruén *et al*., 2020).

Biofilm formation

The formation of biofilm occurs through a series of sequential steps (Figure 3): (a) initial or reversible attachment on the conditioned surface, (b) irreversible attachment, (c) microcolony or early development of biofilm structure, (d) maturation of biofilm, which forms a mushroom- or tower-like structure, and (e) dispersion or detachment, in which cells slough off from the matrix and return to their original free-floating planktonic state (Sharma *et al*., 2019; Oluwole, 2022; Flemming *et al*., 2016; Dufour *et al*., 2010)

a. Initial or Reversible Attachment

Reversible attachment requires an interaction between planktonic microbes and a conditioned surface. This interaction is very weak, involving van der Waals forces, electrostatic forces, and hydrophobic interactions. The attachment works most effectively on surfaces that are rough, hydrophobic, and coated with

a variety of organic compounds. Bacterial features such as fimbriae, pili, and flagella have been hypothesized to lend greater power to the connection between bacteria and the surface. At this stage, bacteria may commit to a biofilm lifestyle or detach and return to the planktonic environment (Sharma *et al*., 2019; Oluwole, 2022; Flemming *et al*., 2016; Dufour *et al*., 2010; Singh *et al*., 2017).

b. Irreversible Attachment

Loosely attached organisms strengthen their hold by producing extracellular polymeric compounds that combine with surface materials and/or receptor-specific ligands on pili, fimbriae, fibrillae, or both. This enables a more secure connection to the host surface. After attaching to preconditioned and permissive surfaces, microorganisms initiate irreversible adhesion and eventually form multilayered cell clusters. During this phase, various physiological and structural changes occur, including the attached cells becoming immobile and losing motility (Sharma *et al*., 2019; Oluwole, 2022; Flemming *et al*., 2016; Dufour *et al*., 2010; Singh *et al*., 2017).

c. Microcolony Formation

Once securely affixed to conducive surfaces, a wide variety of bacteria rise to the surface and release polymeric compounds. These substances act as "glue" to bind microorganisms to surfaces. Following these sequential events, microcolonies form. Microcolony creation results from the simultaneous aggregation and growth of microorganisms, accompanied by the production of extracellular polymeric substances (EPS) (Sharma *et al*., 2019; Oluwole, 2022; Flemming *et al*., 2016; Dufour *et al*., 2010; Singh *et al*., 2017).

d. Biofilm Maturation

Under suitable conditions, biofilms can evolve into spatially ordered, three-dimensional structures such as mushroom- or tower-like formations. Quorum sensing—a density-dependent chemical signaling process in bacterial populations embedded in their self-produced extracellular matrix—mediates this cooperative group behavior, also known as biofilm formation. These signaling mechanisms enable bacteria to communicate, coordinate group activities like virulence factor production and biofilm assembly, and activate coordinated maturation and disassembly of the biofilm (Sharma *et al*., 2019; Oluwole, 2022; Flemming *et al*., 2016; Dufour *et al*., 2010; Singh *et al*., 2017).

e. Biofilm Dispersal

Biofilm formation is a cyclical process in which bacterial cells detach from the mature biofilm and revert to their planktonic state. Detached cells seek new surfaces to colonize, initiating a fresh cycle of biofilm creation. At this stage, individual microbial cells decide—based on environmental signals—whether to remain aggregated or dispersed. Nutrient limitation, for example, compels bacteria to seek new habitats (Sharma *et al*., 2019; Oluwole, 2022; Flemming *et al*., 2016; Dufour *et al*., 2010; Singh *et al*., 2017).

Biofilm Recalcitrance

Resistance

Antibiotic resistance and antibiotic tolerance are two separate phenomena that are included in the category of biofilm recalcitrance (Figure 4). The ability of a bacterium to continue to live and develop in the presence of elevated antibiotic concentrations for extended periods of time is referred to as resistance, and its degree can be measured by determining the minimum inhibitory concentration (MIC). It is possible for horizontal gene transfer (HGT) or mutations to develop the mechanisms that inhibit the binding of an antibiotic to its target. These mechanisms include enzymatic deactivation, active efflux of a medication once it is in the cytoplasm or the cytoplasmic membrane, or reduced inflow, among others. Together, they prevent antibiotics from affecting the function of their target, and they stop the creation of harmful products that would damage the cell if they were allowed to. Additional categories of resistance include intrinsic resistance, acquired resistance, and/or adaptive resistance (Uruén *et al*., 2020; Oluwole, 2022; Flemming *et al*., 2016).

a. *Intrinsic Resistance*

This intrinsic or natural resistance to antibiotics is a characteristic that is present in the bacteria. Because of the presence of the outer membrane, which limits the permeability to many antibiotics, Gram-negative bacteria, for example, are generally more resistant to antibiotics than Gram-positive bacteria are. Another illustration of this phenomenon is the genus of wall-less bacteria known as *Mycoplasma*, which is resistant to

antibiotics whose primary target is the cell wall resistance (Uruén *et al*., 2020; Oluwole, 2022; Flemming *et al*., 2016).

b. *Acquired Resistance*

This occurs as a result of genetic adjustments made to bacteria that were initially sensitive. These modifications can occur as a result of mutations or HGT. Therefore, microorganisms that are initially sensitive to an antibiotic can develop resistance to that antibiotic as a result of spontaneous or induced mutations that change, for example, the target of the antibiotic or its uptake by the cell, or as a result of the acquisition of one or more molecular mechanisms for AMR, such as antibiotic inactivation or increased antibiotic efflux. This can happen when the microorganisms undergo one of these processes. These genetic alterations are inheritable and will have a lasting impact on the organism if the fitness costs associated with them are minimal or nonexistent and there are no compensatory mechanisms of resistance (Uruén *et al*., 2020; Oluwole, 2022; Flemming *et al*., 2016).

c. *Adaptive Resistance*

This refers to the ability of bacteria to swiftly change either the expression of their genes or the production of their proteins in response to drugs or unfavorable environmental conditions. The epigenetic inheritance, population heterogeneity, gene amplification, and efflux pumps that are regulated by sophisticated regulatory pathways are all components of the molecular process that underlies this resistance phenomenon (Uruén *et al*., 2020; Oluwole, 2022; Flemming *et al*., 2016).

d. Heteroresistance

This refers to the occurrence of one or more subpopulations, within a given population of bacteria, that demonstrate elevated levels of antibiotic resistance in comparison with the main population. This behavior is frequently linked to the presence of unstable genes within the bacteria, which would provide the organism with a high probability of reverting back to susceptibility in the absence of antibiotic selective pressure. Due to its instability, discovery is difficult, which in turn increases the likelihood that treatment will be unsuccessful resistance (Uruén *et al*., 2020; Oluwole, 2022; Flemming *et al*., 2016).

Tolerance

Antibiotic tolerance refers to bacteria's ability to withstand temporary exposure to elevated antibiotic doses—even above the minimum inhibitory concentration (MIC)—measured by the minimum bactericidal concentration (MBC) needed to kill 99.9% of cells; unlike resistance, tolerance is transient, with prolonged exposure eventually killing the bacteria through adaptive shifts from active growth to a quiescent, dormant state involving major metabolic rearrangements like altered energy production and non-essential functions under unfavorable growth conditions, stress, or antibiotics, where targets remain accessible but non-essential. Biofilm antibiotic tolerance also arises from ECM entrapment preventing antibiotics from reaching infection sites, with tolerant cells unable to grow under bactericidal antibiotics unlike resistant ones; tolerance represents a specific instance of persistence, which increases population survival against bactericidal antibiotics without raising MIC, affecting only a subpopulation of persister cells that endure repeated exposures, categorized as type I (triggered by environmental signals like starvation) or type II (spontaneous stochastic conversion in growing populations), also termed heterotolerance—distinct from heteroresistance—as persisters susceptible to extended exposures. Overall, biofilm recalcitrance stems not from a single mechanism but a synergy of tolerance processes and resistance mechanisms, varying by bacterial species/strain, antimicrobial agent, biofilm developmental stage, and growth conditions (Uruén *et al*., 2020; Oluwole, 2022; Flemming *et al*., 2016).

Mechanism of Biofilm resistance to Antimicrobial Agents

a. Capsules or Glycocalyx

Both gram-positive and gram-negative bacteria possess glycocalyx in their biofilms, with thicknesses ranging from 0.2 to 1.0 micrometers, serving as an essential component that employs electrostatic, Van der Waals, and hydrogen bond forces to promote biofilm cohesion and adherence to solid surfaces, thereby aiding maturation. Pathogenic bacteria endure hostile host environments through glycocalyx composition adaptability and regulation, integrated with biofilm formation, where environmental variables influence biofilm capsule components like glycoproteins and polysaccharides, which are condition-regulated. The glycocalyx

matrix bolsters bacterial resistance to antibiotics and antimicrobial treatments by absorbing up to 25% of its weight in antimicrobial molecules, with adsorption sites acting as barriers to biocide penetration and anchors for exoenzymes that hinder certain antibacterial drug motility, supply substrates for biocide metabolite degradation, and diminished activity of exoenzyme-vulnerable pharmaceuticals. (Uruén *et al*., 2020; Sharma *et al*., 2019; Flemming *et al*., 2016).

b. Enzyme-Mediated Resistance

Enzymes that offer resistance to biofilm are responsible for the transition of bactericide into its harmless form. Very few species of bacteria have been described as being capable of degrading hazardous chemicals such as aromatic and phenolic compounds, as well as other heavy metals such as nickel, cadmium, mercury, antimony, silver, copper, zinc, lead, and cobalt, among others. Enzymatic reduction of ions and metal resistance genes are the typical mechanisms behind the detoxification process. The presence of heavy metals led to the development of a more resistant phenotype throughout a wider spectrum (Uruén *et al*., 2020; Sharma *et al*., 2019; Flemming *et al*., 2016).

c. Heterogeneity in Metabolism and Growth Rate

Adaptive diversity within bacterial populations increases the probability that some individuals can address immediate or future challenges, as variations in nutrient and oxygen levels within biofilms influence bacterial development rates and metabolic activity, with differing concentrations of metabolic substrates and products indicating varying growth and activity levels, exemplified by Clostridia providing insights into growth and fermentation across cultivation conditions, thereby fostering microbial community diversity. In biofilm outer layers, abundant nutrients and oxygen boost cellular metabolism and bacterial proliferation, whereas poor nutrient diffusion deeper inside restricts metabolic potential, slowing growth via guanine nucleotide-guanosine 3,5'-bis-pyro-phosphate (ppGpp) accumulation and reduced tRNA/rRNA synthesis, while cellular enzyme production reflects this metabolic and growth rate heterogeneity, fluctuating with growth cycles proportional to cell mass and halting in stationary or slow-growth phases. Latent-phase bacteria exhibit reduced susceptibility to antimicrobials—despite biocides targeting active cells—as *E. coli*'s RelA-dependent ppGpp inhibits autolysin activity and caps anabolic processes during dormancy; relA mutants, without growth rate changes, show increased antibiotic sensitivity due to impaired peptidoglycan production, diminishing cell wall inhibitor efficacy, thus reinforcing how metabolic growth rate heterogeneity underpins biofilm resistance. Oxygen regulates biofilm metabolism, eradicating *Pseudomonas aeruginosa* biofilms with ciprofloxacin and tobramycin under pure oxygen, while reducing oxygen elevates resistance, augmented by anaerobic gene expression (Uruén *et al*., 2020; Sharma *et al*., 2019; Flemming *et al*., 2016).

d. *Phenomenon of Persistence Shown by Cells*

Persister cells, a subpopulation resistant to antimicrobial agents, are responsible for severe chronic infections and pose significant challenges in identifying diverse bacterial strains clinically. Research on ATP-dependent persister formation shows that reduced ATP levels diminish antibiotic target activity, leading to persister development, while bacterial biofilms harbor multidrug- and bactericidal-resistant persister cells, particularly in late-growing gram-positive or gram-negative bacteria exhibiting tolerance or persistence. Persister generation aligns with bacterial growth stages, enabling rapid propagation and survival under lethal antimicrobial concentrations, with stationary-phase bacteria producing high persister numbers linked to increased biofilm resistance; these persisters survive post-antibiotic treatment in planktonic populations after immune clearance, shielded by the glycocalyx matrix, and reinitiate biofilm formation upon antibiotic cessation in sessile populations, although stationary-phase dilution disadvantages them. Persister formation depends on bacterial metabolic activity, suggesting they represent a dormant wild-type state rather than mutants unresponsive to bactericidal drugs, competing for antibiotic targets essential for multidrug resistance (MDR) protein production—emphasizing that antibiotics disrupt cellular functions rather than merely inhibiting growth, linking to cell damage and persistent tolerance phenomenon tied to programmed cell death (PCD), where antimicrobials induce damage without full lysis, indirectly triggering PCD via autolysis mediated by biofilm peptidoglycan hydrolases (autolysins). Recent studies on late-growing bacteria using modified Kirby-Bauer disk diffusion tests—swapping nutrient-loaded antibiotic discs for antibiotic-free controls—assess resistance and tolerance levels (Uruén *et al*., 2020; Sharma *et al*., 2019; Flemming *et al*., 2016).

e. Metabolic State of the Organisms in the Biofilm

Biofilm-specific growth within biofilms has been proposed as a key explanation for resistance, with substantial bacterial susceptibility variance to bactericides arising from physiological cell states and environmental factors; nutrient limitation alters barrier composition and bacterial cell membranes, fostering phenotypic adaptation where resistant populations emerge post-bactericide treatment at growth-inhibiting concentrations. Stress from heat or starvation induces *E. coli* resistance to H2O2 and UV light, while Enterococcal strains upregulate antioxidative enzymes and downregulate prooxidative ones under oxidative stress, although the resistant phenotype vanishes upon bactericide removal. Nutrient deficiency in biofilms stalls growth, inducing a starved state that diminishes antimicrobial efficacy at the biofilm-bulk fluid interface, impairing respiratory action, whereas non-growing cells in fast-growing, nutrient-rich media exhibit reduced susceptibility to various antimicrobials (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

f. Genetic Adaptation

Genetic adaptation within biofilms is essential for reducing susceptibility and adopting a reasonably protected, distinct phenotype. In *E. coli*, multiple antibiotic resistance (mar) operons act as global regulators, controlling numerous genes to support a multidrug-resistant phenotype encompassing resistance to antibiotics, organic solvents, and disinfectants. *M. tuberculosis* can enter a dormant state persisting for decades in hostile environments, evading anti-TB drugs, with most bacteria exhibiting fermentative metabolism, oxidant-repair/degrading enzymes, and oxidative stress responses that enhance resistance after brief exposure to sub-inhibitory stressor concentrations. In *E. coli*, identified defense genes encode catalysts such as superoxide dismutases, hydroperoxide reductases, and alkyl glutathione reductases, alongside DNA repair enzymes; exposure to oxidants activates regulatory genes such as oxyR and soxR, which modulate intracellular redox potential and trigger stress responses (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

g. Quorum Sensing (Cell to Cell Signaling)

Quorum sensing (QS) is a cell-to-cell communication process in bacteria that regulates behavior through extracellular signal molecules, including their detection, synthesis, and autoinducers. Studies like those by Reboule *et al*. explore natural compounds that block QS by acting as agonists against receptors, enabling bacteria to detect increased population density and activate specific gene groups in response. In gram-negative bacteria, QS involves producing and secreting acyl homoserine lactones (AHL) that diffuse through the cell wall into the medium; in gram-positive bacteria, it relies on secreted peptides detected via two-component systems (membrane-bound histidine kinase receptor and intracellular response regulator) to alter gene expression patterns. Autoinducers-2 represent another QS mechanism utilized by both gram-positive and gram-negative bacteria to varying extents, while the glycocalyx matrix and degradative enzymes—regulating signaling molecule production from precursors like S-adenosyl methionine and acyl-carrier proteins—influence active cells in bacterial growth, with QS also playing a key role in controlling biofilm maturation (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Talagrand-Reboul E *et al*., 2017).

Signal molecule-mediated quorum sensing plays a crucial role in biofilm production across diverse bacterial species, influencing biofilm architecture and regulating degradative enzyme production. Under adequate nutrients and suitable environments, quorum sensing-mediated phenotype expression is vital for cell migration and protects novel growth modes from environmental harm. In *Aeromonas*, three QS systems regulate biofilm formation, motility, and virulence, while in *Pseudomonas aeruginosa*, cell-to-cell signaling controls superoxide dismutase and catalase genes, enhancing hydrogen peroxide resistance. Defective or mutant biofilms lacking quorum sensing produce less extracellular polymeric substance (EPS), resulting in thinner structures more susceptible to kanamycin, with quorum sensing responding to environmental stress directly or indirectly via biofilm. Studies on *P. aeruginosa* show it eliminates competitors using the 2-heptyl-3-hydroxy-4-quinolone signal, which exploits stored iron; prior research on *Pseudomonas* quinolone signals indicates higher affinity of iron chelators, and Schertzer *et al*. found similarities between glycocalyx matrix/signal molecule activity in capturing externally positively charged substances (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017; Schertzer JW *et al*., 2009).

h. Stress Responses

Biofilms exhibit a stress response involving physiological and morphological changes that enhance bacterial cellular stress resistance. This response generally controls cell membrane formation and thin aggregative fimbriae production in *Salmonella enterica serovar Typhimurium* and *E. coli*, acting primarily to prevent cellular damage rather than repair it. Stress induction arises from factors such as nutrient restriction during stationary-phase growth, high/low temperatures, elevated osmolality, and acidic pH; Notably, *E. coli*'s sigma subunit of RNA polymerase, RpoS, activates under adverse conditions, regulating 50 genes for stress tolerance while others drive physiological rearrangements or metabolic redirection in response to stressors. Increased biocide resistance may emerge from general stress response-induced gene expression changes in cells trapped within the biofilm matrix (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

i. Outer Membrane Structure

Antibacterial drug efficacy requires penetration, leading bacterial cells to develop antibiotic resistance by modifying cell envelopes or adapting to environments. The lipopolysaccharide layer and underlying phospholipids primarily block hydrophilic agents from crossing the outer membrane, while outer membrane proteins exclude hydrophobic ones. Gram-positive bacteria like *Nocardia farcinica* feature complex cell walls binding diverse lipids and proteins that form pores and hydrophilic pathways interacting with antibiotics. Drug-resistant mycobacterial strains depend on intact outer membranes; some resistant bacteria lack or overexpress outer membrane proteins, such as *P. aeruginosa* strains missing OprD porin—selective for specific carbon sources and isothiazolone entry—while altered outer membrane protein profiles exclude bactericides like sodium dimethyl dithiocarbonate (SMT) (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

j. Efflux Pumps

Efflux systems enable bacterial survival in harsh antimicrobial environments by expelling drugs, conferring both innate and acquired resistance to agents from the same or different families, with overproduction linked to multidrug resistance (MDR) that synergizes with mechanisms like targ*et al*teration and antibiotic inactivation. *Acinetobacter baumannii* poses hospital challenges through antibiotic resistance, environmental persistence, and wound biofilm formation, while gram-positive pathogens like *Bacillus spp., Lactobacillus*, and *Staphylococcus aureus* cause severe infections via diverse resistance mechanisms and transport of unrelated substances, resulting in MDR phenotypes. Efflux pumps in pathogens such as *E. coli*, Enterobacter aerogenes, and *Klebsiella pneumonia*e hinder hydrophilic solute penetration and reduce lipophilic solute transmembrane diffusion by downregulating porin protein production; genes encoding these pumps localize on chromosomes or plasmids, mediating resistance to antibiotics, biocides, dyes, and detergents (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

The major facilitator superfamily (MF), the resistance-nodulation-division family (RND), the small multidrug resistance family (SMR), the ATP-binding cassette family (ABC), and the multidrug and toxic compound extrusion family are the five distinct classes of bacterial efflux pumps that have been discovered (MATE). The ABC family system breaks down ATP in order to drive antimicrobial agent efflux. The MF family, the MATE family, and the RND family all operate as secondary transporters and catalyze drug ion antiprotons (H+ or Na+). Transporters belonging to the RND family are the first line of defense in bacteria because they serve as targets for mutations or pharmacological modifications (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

Expression of multi-drug resistance operons and efflux pumps is triggered in bacterial biofilms exposed to subinhibitory antibiotic doses such as chloramphenicol and tetracycline, or xenobiotics such as salicylate and chlorinated phenols. DNA microarray analysis of mature *Pseudomonas aeruginosa* PAO1 biofilms revealed no activation of RND efflux system genes in sessile populations grown in antibiotic-free conditions. In *E. coli* biofilms, mar and acrAB encoding genes regulate the multidrug resistance phenotype; antibiotics penicillin, cephalosporin, rifampicin, nalidixic acid, fluoroquinolones, and oxidative stress factors upregulate mar in planktonic bacteria, inducing resistance, while sub-lethal doses of tetracycline, chloramphenicol, salicylate, and paracetamol further elevate mar expression (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

In addition to being described as being up regulated in mar mutants, the acrAB efflux pump was responsible for determining the multidrug resistance phenotype of mar mutant isolates. Constitutive expressions of the acrAB efflux pump are responsible for the enhancement of a lower susceptibility of *E. coli* biofilm to sub-lethal dosages of ciprofloxacin. In addition, the expression of mar and the genes that it targets is connected to the period of bacterial growth known as the stationary phase. In the case of *E. coli* biofilm, it was shown that a higher expression level of mar led to a significant reduction in the metabolic activity of the bacteria. The susceptibility of *Pseudomonas aeruginosa* biofilm to antibiotics is significantly reduced in the presence of mutants of *Pseudomonas aeruginosa* that have enhanced expression of efflux pumps. Efflux pumps are the primary cause of multidrug resistance in gram-negative bacteria and represent one of the most significant challenges in the field of drug discovery. Recent developments in our knowledge of efflux pumps may offer the foundation for drug discovery in bacterial systems (C. Mah TF *et al*., 2001;Dufour D*et al*., 2010; Hall CW *et al*., 2017).

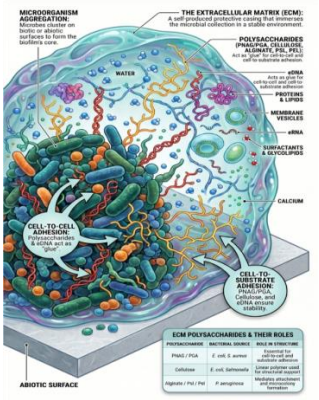

Fig. 1. Detailed structure of the extracellular matrix (ECM) in a mature biofilm

This schematic illustrates a complex biofilm community, featuring a central aggregation of diverse microbial cells (in blue, green, and orange shades) forming the core attached to biotic or abiotic surfaces. Enveloped by a self-produced extracellular matrix (ECM) for protection and stability, key components include polysaccharides (e.g., PNAG/PGA, cellulose, alginate, PSL, PEL) as primary "glue" for cell-to-cell and cell-to-substrate adhesion; reinforcing eDNA (red helices); plus eRNA, proteins & lipids, membrane vesicles, surfactants & glycolipids, calcium ions, and water channels. An insert table links specific polysaccharides (PNAG/PGA, cellulose, alginate/Psl/Pel) from bacteria like *E. coli, S. aureus, Salmonella*, and *P. aeruginosa* to roles in adhesion, support, and microcolony formation. This figure was made using Notebooklm.

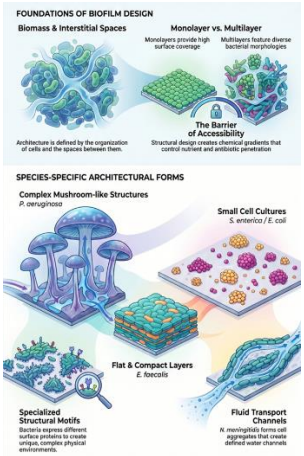

Fig. 2. Overview of Biofilm Architecture

This figure depicts how spatial organization in bacterial biofilms drives community function and resilience. The top section on "Foundations of Biofilm Design" outlines architectural elements like biomass and interstitial spaces, contrasting high-coverage monolayers with multilayer structures that feature diverse cells, chemical gradients, and a "barrier of accessibility" impeding nutrients and antimicrobials. The bottom

section illustrates species-specific forms: mushroom-like structures in *P. aeruginosa* for nutrient flow; dense, flat layers in *E. faecalis*; simple clusters in *S. enterica* or *E. coli*; and fluid channels in *N. meningitidis* for hydraulic efficiency. Bacteria also use surface proteins to craft specialized motifs, optimizing local environments within the matrix. This figure was made using Notebooklm.

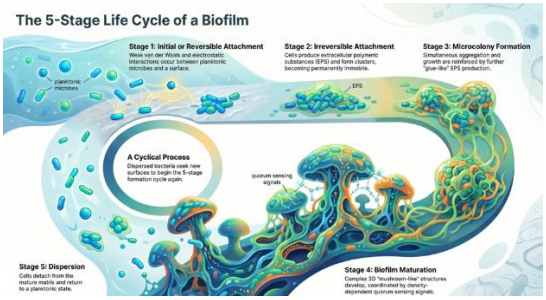

Fig. 3. The cyclical five-stage life cycle of a bacterial biofilm

This diagram outlines the five progressive stages of biofilm development. Stage 1 involves reversible attachment of planktonic microbes to surfaces via weak forces. In Stage 2, irreversible attachment occurs as cells produce EPS and form immobile clusters. Stage 3 features microcolony formation with "glue-like" EPS enabling growth and aggregation. Stage 4 marks maturation into complex 3D "mushroom-like" structures coordinated by quorum sensing signals. Finally, Stage 5 is dispersion, where cells revert to planktonic form to colonize new surfaces and restart the cycle. This figure was made using Notebooklm.

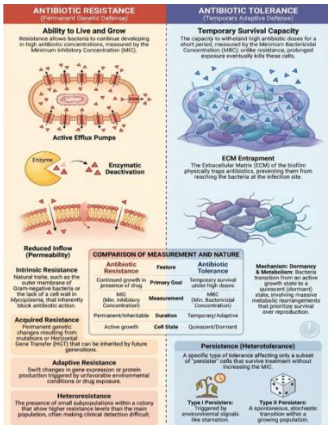

Fig. 4. Conceptual framework distinguishing antibiotic resistance and tolerance as distinct mechanisms contributing to biofilm recalcitrance

This schematic depicts biofilms' dual defenses against antibiotics: permanent genetic antibiotic resistance (measured by MIC), including efflux pumps, enzymatic deactivation, and reduced permeability—categorized as intrinsic, acquired, adaptive, or heteroresistance; and temporary antibiotic tolerance (measured by MBC), via ECM entrapment and metabolic dormancy (persisters: Type I starvation-induced or Type II spontaneous). A central table contrasts their features, metrics, and cell states. This figure was made using Notebooklm.

IV. CONCLUSION

This study demonstrated that biofilms represent a highly virulent bacterial lifestyle and play a major role in the increasing burden of antimicrobial resistance through a combination of physical, physiological, and genetic mechanisms. The extracellular matrix serves as the primary protective barrier that limits antibiotic penetration, while metabolic heterogeneity, the presence of persister cells, quorum sensing, outer membrane modification, and efflux pump activity further enhance bacterial survival against therapeutic interventions. The main findings confirmed that biofilm recalcitrance is not caused by a single factor, but rather by a dynamic interaction between genetically mediated resistance and phenotypic tolerance. Therefore, conventional single-antibiotic therapeutic approaches are often insufficient for the eradication of chronic biofilm-associated infections. Practically, these findings imply the need for integrated clinical strategies involving combinations of antibiotics, biofilm matrix-disrupting agents, quorum sensing inhibitors, and early

detection systems on medical devices and hospital environments to reduce treatment failure rates and the spread of nosocomial infections.

However, this study had several limitations because it employed a literature review approach and was therefore highly dependent on the quality, scope, and heterogeneity of previous studies, while lacking direct experimental verification using specific clinical isolates. In addition, most available sources were dominated by in vitro models that did not fully represent in vivo conditions such as host immune responses, nutrient gradients, and the complexity of infected tissues. Therefore, future research should develop experimental and translational studies based on multi-omics, metagenomics, and animal or organoid models to better understand the relationship between biofilm architecture and resistance gene expression. Further studies should also evaluate natural and synthetic antibiofilm compounds, nanoparticle technologies, and the integration of artificial intelligence for predicting biofilm formation and personalizing therapy. These efforts are essential to ensure that biofilm-associated infection control can be achieved more effectively, precisely, and sustainably in the future.

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