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Microbial biofilms: molecular mechanisms of antibiotic resistance and methods for their investigation

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Abstract. *By performing a literature review, to summarize current evidence on the molecular mechanisms underlying biofilm-associated resistance to antibacterial agents and to analyze laboratory approaches used for biofilm cultivation, visualization, and antimicrobial susceptibility assessment. An analytical review of Ukrainian and international publications on biofilms, antimicrobial resistance, and biofilm research methods was performed. Sources were identified through open scientific databases and library resources, including PubMed, Google Scholar, ScienceDirect, SpringerLink, Scopus, and domestic library collections. The search strategy included terms related to biofilms, bacterial biofilms, antimicrobial resistance, antibiotic tolerance, molecular resistance mechanisms, biofilm research methods, and biofilm-associated infections. The review demonstrates that biofilm resistance is a multifactorial phenomenon generated by the combined action of several protective layers. A central role belongs to the extracellular matrix, which contains exopolysaccharides, proteins, lipids, and extracellular DNA and acts as both a structural scaffold and a diffusion barrier. Biofilm tolerance is further reinforced by marked spatial and metabolic heterogeneity, limited antibiotic penetration into deeper layers, the emergence of dormant persister cells, reduced growth activity, efflux-mediated drug removal, enzymatic antibiotic inactivation, target modification, and dissemination of antibiotic resistance genes. Together, these factors markedly decrease the susceptibility of biofilm-embedded microorganisms compared with planktonic cells. The paper also systematizes the main laboratory models used to study biofilms. Static systems, including tube assays, Congo Red Agar, and microtiter plate methods, are suitable for primary screening and quantitative biomass assessment, but only partially reproduce the gradients and hydrodynamic conditions typical of natural and clinical environments. Dynamic models, such as flow-cell systems, drip-flow reactors, rotating disk reactors, and CDC biofilm reactors, allow mature biofilms to be formed under controlled flow and shear conditions, although they require more complex instrumentation. Special attention is given to methods for testing biofilm susceptibility, including CFU counting, MBEC-based approaches, MBC-B determination, and metabolic viability assays, which provide a more informative comparison of antibiotic activity against planktonic and biofilm populations. Biofilm-associated resistance cannot be attributed to a single determinant, since it arises from the interaction between matrix-mediated protection, physiological heterogeneity, adaptive phenotypic states, and genetic resistance mechanisms. Existing in vitro models have significantly improved the study of biofilm biology and antimicrobial susceptibility, yet none is universally applicable to all research and clinical tasks. Further development of standardized and reproducible methods that retain ecological and clinical relevance is essential for better diagnostics and for designing more effective strategies against biofilm-associated infections.*

Keywords: biofilms; antibiotic resistance; antibiotic tolerance; extracellular matrix; persister cells; MBEC; MBC-B; biofilm research methods

The microbial world occupies a unique place in the system of living nature. Despite the relative limitation of morphological forms, microorganisms possess numerous mechanisms that enable survival under almost any environmental conditions, allowing them to readily withstand adverse effects and occupy all known ecological niches.

Unicellular microorganisms exhibit two behavioral strategies: free movement or swimming of individual cells in a liquid medium, and a stationary state in which cells are tightly attached to a surface and connected to one another in a single structure (a community, the so-called biofilm) (Zhao, 2023).

Until the end of the last century, it was traditionally believed that all bacteria under natural conditions existed as free-floating planktonic cells, and all knowledge regarding bacterial morphology, physiology, genetics, and adaptive capacities had been obtained from the study of bacterial populations formed by individual cells or colonies cultured from a single cell.

With the development of microscopic methods and molecular genetic technologies, evidence was obtained that natural bacterial populations may also exist in the form of substrate-attached biofilms, within which bacteria exchange biochemical signals and exhibit coordinated activity similar to that of multicellular organisms (Zhao, 2023; Vestby, 2020).

Aim of the study. The aim of this review was to systematize current data on the molecular mechanisms of antibiotic resistance in biofilms and to critically analyze the laboratory models used for their investigation.

Materials and methods. This study analyzed scientific publications by Ukrainian and foreign authors, including the results of experimental, clinical, and review studies. Information sources were selected from open online research databases, including PubMed, Google Scholar, ScienceDirect, SpringerLink, and Scopus, as well as domestic library resources. For targeted literature retrieval, the following keywords and phrases were used: “biofilms,” “bacterial biofilms,” “antimicrobial resistance,” “antibiotic tolerance,” “molecular mechanisms of resistance,” “biofilm research,” “methods for studying biofilms,” and “biofilm-associated infections.”

Results. *Biofilms: structure and stages of development.* According to current concepts, a biofilm is a continuous layer of bacterial cells that are firmly connected to one another and to a surface by means of a biopolymeric extracellular matrix. Such bacterial structures are formed by one or several bacterial species and include both actively functioning cells and inactive (resting, dormant) forms. Biofilms may consist of commensals as well as representatives of the opportunistic and pathogenic microflora of the skin, nasopharynx, intestine, and other human microbiota (Zhao, 2023; Koshova, 2025).

Biofilms are of extremely high clinical importance because they participate in the pathogenesis of numerous bacterial infections that are difficult to treat with antibiotics (Vestby, 2020; Schulze, 2021). For example, *Pseudomonas aeruginosa* biofilms cause chronic pulmonary infections in patients with cystic fibrosis (Singh, 2021; Thi, 2020; Oluyombo, 2019). Their ability to colonize the surfaces of medical devices used for implantation and catheterization (catheters, implants, endoprostheses, etc.) also creates substantial therapeutic difficulties. For example, *Staphylococcus aureus* biofilms can colonize medical devices such as pacemakers. Fungal and bacterial populations often form mixed-species biofilms, which further complicates the treatment of polymicrobial infections (Schulze, 2021; Singh, 2021; Stewart, 2020; Caldara, 2022).

The complex architecture of biofilms enables metabolic cooperation among cells within the system, creates conditions favorable for symbiotic interactions between microorganisms of different species, and provides protection against external factors (Zhao, 2023; Grooters, 2024).

Biofilm formation is one of the survival strategies of bacteria in the ecological niches they occupy. The biofilm-associated attached state protects bacteria from harmful environmental factors, antibacterial agents, and immune surveillance. In response to changes in temperature, pH, and osmolarity, biofilms undergo morphological differentiation accompanied by changes in microbial metabolism, hydrodynamics, and communicative interactions within the biofilm (Zhao, 2023).

Due to the extracellular matrix, microorganisms organized within biofilms are significantly less susceptible to antimicrobial agents than planktonic (free-floating) cells. As a result, infections associated with biofilm formation are extremely difficult to cure. The high level of resistance characteristic of biofilms is promoted by a broad spectrum of molecular mechanisms, including, among others, interactions of antimicrobial agents with components of the biofilm matrix, reduced growth rate, and modifications in the action of specific genetic determinants of antibiotic resistance and tolerance. Individually, each of these mechanisms explains only part of the enhanced antimicrobial resistance observed in biofilms. However, acting in concert, these protective mechanisms help ensure survival of biofilm cells even under the most aggressive antimicrobial treatment regimens (Mishra, 2024). The phenotypic transition from free-swimming planktonic lifestyle to sessile existence within a biofilm is a tightly regulated developmental process dependent on numerous ecological and genetic factors that vary by species (Zhao, 2023; Muhammad, 2020).

According to the classical biofilm development model, at the initial stage motile planktonic cells attach to a surface in response to various environmental signals. For example, one of many signals capable of inducing biofilm formation in *P. aeruginosa* and *E. coli* is exposure to subinhibitory concentrations of aminoglycosides. Attached cells produce a hydrated matrix composed of exopolysaccharides, extracellular DNA (eDNA), proteins, and lipids. Over time, sessile cell groupings in microcolonies mature into macrocolonies. Cells may leave mature biofilms to return to the planktonic state and subsequently colonize new surfaces (Campoccia, 2021; Panlilio, 2021).

Biofilm formation is a complex, multistage, tightly regulated process. Individual members of the developing community have their own “specific duties,” which collectively enhance the viability of the entire community. Intercellular communication in a biofilm is mediated by a mechanism known as quorum sensing. Information exchange occurs through special chemical signaling molecules, autoinducers, by means of which the microbial community acts as a single organism (Priya, 2023).

Biofilms go through five stages in their development (Zhao, 2023; Singh, 2021; Grari, 2025):

1. At the first stage of biofilm formation, primary adhesion (sorption, attachment) of microorganisms from the environment to a given surface occurs. This process is reversible and is determined by physicochemical interactions between the microbial cell envelope and the substrate, including electrostatic interactions, van der Waals forces, and hydrophobicity. Surface adhesins (biofilm-associated proteins), teichoic acids of Gram-positive bacteria, and environmental factors, including pH, ionic strength, and salt concentration, play an important role. Hydrodynamic conditions also substantially influence the course of adhesion: under low shear stress, cells attach more easily, whereas intense flow may cause erosion of attached cells or hinder their fixation (Ciofu, 2022).
2. At the second stage, the adhered microorganisms become fixed on the surface through secretion of extracellular polymers that ensure stronger adhesion. The mucopolysaccharide layer produced by microorganisms immediately after adhesion protects them from the surrounding environment and constitutes a factor of biofilm resistance. This is the final (irreversible) attachment stage (Grari, 2025).
3. At the maturation stage, attached bacteria begin to actively produce the extracellular polymeric substances (EPS) matrix, which holds cells together and ensures the spatial stability of the colony. The matrix creates a three-dimensional scaffold within which nutrients accumulate, microcolonies are formed, and internal physiological differentiation of cells arises. Enhanced adhesion and intercellular interactions promote coaggregation, a process by which new cells integrate into an existing structure. Cell movement across the surface also plays a substantial role in biofilm growth. Among the described types of such movement are swarming motility, mediated by flagella and biosurfactants; twitching motility, associated with type IV fimbrial contractions; and gliding motility, which ensures slow sliding of cells across a solid substrate. These forms of surface translocation contribute to consolidation of microcolonies and to the transition of the biofilm toward mature three-dimensional architecture (Grari, 2025).
4. At the growth stage, the biofilm develops into a mature three-dimensional structure that increases in volume, becomes architecturally more complex, and acquires characteristic morphology. This stage includes active synthesis of extracellular matrix components and formation of spatially organized microcolonies. The biofilm develops typical architectural patterns such as “mushroom-shaped” or “pyramidal” structures (Fig. 1G), between which channels for fluid circulation are formed. This organization ensures effective distribution of nutrients, oxygen, and signaling molecules among all cell clusters. Polysaccharides involved in intercellular adhesion and other EPS components play an important role in maintaining structural integrity by ensuring cohesion and coordinated interactions among bacterial cells (Quan, 2022).
5. The dispersion stage is characterized by partial disruption of the biofilm and release of cells into the surrounding environment. After maturation is completed, individual bacteria or small cellular aggregates detach from mature microcolonies and migrate to new surface areas with suitable conditions for formation of secondary biofilm structures. Dispersion is promoted both by active mechanisms (changes in bacterial motility, including twitching motility) and passive pro-

cesses associated with degradation of the extracellular matrix. Various bacterial enzymes participate in the disruption of the EPS structure, including alginate lyase (*P. fluorescens*, *P. aeruginosa*), N-acetylheparosan lyase (*E. coli*), hyaluronidase (*Streptococcus* spp.), as well as surface surfactants. These enzymes degrade matrix components, including polysaccharides, eDNA, proteins, and other polymeric structures, thereby weakening intercellular connections and enabling cell release. As a result, microorganisms within the biofilm can colonize new ecological niches (Sajna, 2021).

Figure 1 schematically presents the stages of biofilm development.

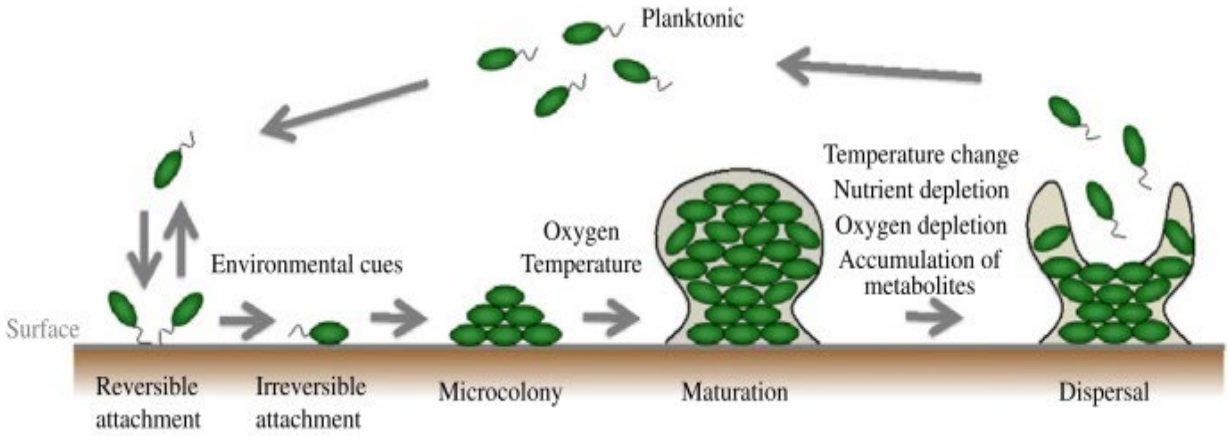


Fig. 1. Stages of biofilm development: Biofilm development includes five sequential stages: reversible attachment, irreversible attachment, microcolony formation, maturation, and dispersal (Mistrologics**,** 2018)

It is generally recognized that the basis of biofilm resistance and tolerance to antibiotics is multifactorial, and the implementation of the corresponding mechanisms depends on the type of antimicrobial agent, the bacterial species and strain, the age and developmental stage of the biofilm, as well as its growth conditions (Patil M., 2025). No single mechanism can explain the increased antibiotic resistance characteristic of biofilms; however, collectively these factors substantially limit the effectiveness of treatment of infections caused by biofilm-forming microorganisms.

Therefore, understanding the mechanisms underlying biofilm antimicrobial resistance is critically important for the development of effective treatment strategies. This review summarizes historical and current data on the mechanisms of resistance and tolerance of biofilm-forming microorganisms and on current technologies for studying bacterial biofilms.

Molecular mechanisms of biofilm resistance to antibiotics. Resistance to antimicrobial agents in biofilms is a multifaceted phenomenon involving several mechanisms that together contribute to the increased resistance of bacteria embedded within the biofilm. These microorganisms have been shown to be 10–1000 times more resistant to antimicrobial agents than planktonic cells. Such resistance is a collective property of the microbial community, formed due to the unique EPS structure, metabolic heterogeneity, the emergence of persister cells, and the circulation of genetic determinants of resistance. It is precisely the combination of these factors that determines the high level of tolerance of biofilms to therapy, even under conditions of high concentrations of antimicrobial agents.

The extracellular biofilm matrix is regarded as an anionic polymeric complex whose composition and structure vary among bacterial species. EPS is primarily composed of homo- and heteropolysaccharides containing uronic acids (predominantly glucuronic acid) and amino sugars. A characteristic example is the alginate of *Pseudomonas aeruginosa*, a copolymer of mannuronic and glucuronic acids (Grari, 2025). The exopolymer of *Escherichia coli* contains colanic acid. Various enterobacterial species produce cellulose, which, for example, together with fimbriae, is the main component of *Salmonella* biofilms (Lebeloane, 2024).

In addition to polysaccharides, the matrix contains proteins, lipopolysaccharides, lipids, lectins, other organic components, and minerals (Singh, 2021; Kreve, 2021).

An important structural element is extracellular DNA, which is formed mainly as a result of cell autolysis and creates a mesh-like scaffold that stabilizes the three-dimensional biofilm structure and serves as a source of genetic material for horizontal transfer and as an energy source during cellular starvation (Panlilio, 2021).

Together, these components ensure the mechanical integrity of the biofilm, protection against adverse environmental conditions (UV radiation, radiation, pH changes, osmotic stress, desiccation), and the sorption and concentration of nutrients, enzymes, and growth factors (Wang, 2020).

In addition, biofilms are characterized by marked physiological heterogeneity, and their transcriptomes and proteomes differ from those of planktonic cells, leading to substantial phenotypic differences (Schulze, 2021; Singh, 2021). At the same time, unlike a planktonic culture, which exists under relatively homogeneous environmental conditions, cells within a biofilm are exposed unevenly to nutrients, oxygen, and toxins due to differences in their spatial localization. The combination of these factors with physiological heterogeneity results in the formation of a population of slow-growing or dormant persister cells that are more resistant to antibiotics whose mechanism of action targets active cells (Grari, 2025; Verma, 2023; Singh, 2022).

Unlike genetically determined resistance, persistence is a temporary, non-heritable phenotypic state. Persister cells do not proliferate in the presence of antibiotics; however, after antibiotic concentrations decline, they are able to recolonize the biofilm, thereby contributing to relapse and/or chronification of biofilm-associated infections. As noted by E. Galdiero and co-authors, this form of phenotypic tolerance constitutes a serious therapeutic problem because it allows microorganisms to survive even under antimicrobial pressure (Galdiero, 2020).

At the same time, subpopulations within biofilms exhibit exceptional resistance to host immune responses and antimicrobial therapy (Grande, 2020; Shree, 2023; Moser, 2021; Cangui-Panchi, 2023; Coenye, 2022; Stewart, 2022). For example, *P. aeruginosa* biofilms growing on urinary catheters are approximately 1000 times more resistant to tobramycin than planktonic cells (Koshova, 2024).

Classical mechanisms of antibiotic resistance include antibiotic destruction or modification (in particular, β-lactamases that degrade β-lactam drugs), active efflux of the drug from the cell via efflux pumps, and alteration of the antibiotic target, which reduces its affinity. These strategies are implemented through antibiotic resistance genes (ARGs), which encode the corresponding enzymes, transport systems, or modified target proteins. ARGs may be localized both on the chromosome and on plasmids, which facilitates their horizontal transfer among microorganisms and confers resistance to several major classes of antibiotics, including tetracyclines, sulfonamides, β-lactams, macrolides, lincosamides, streptogramin B, aminoglycosides, and others (Uruén, 2020).

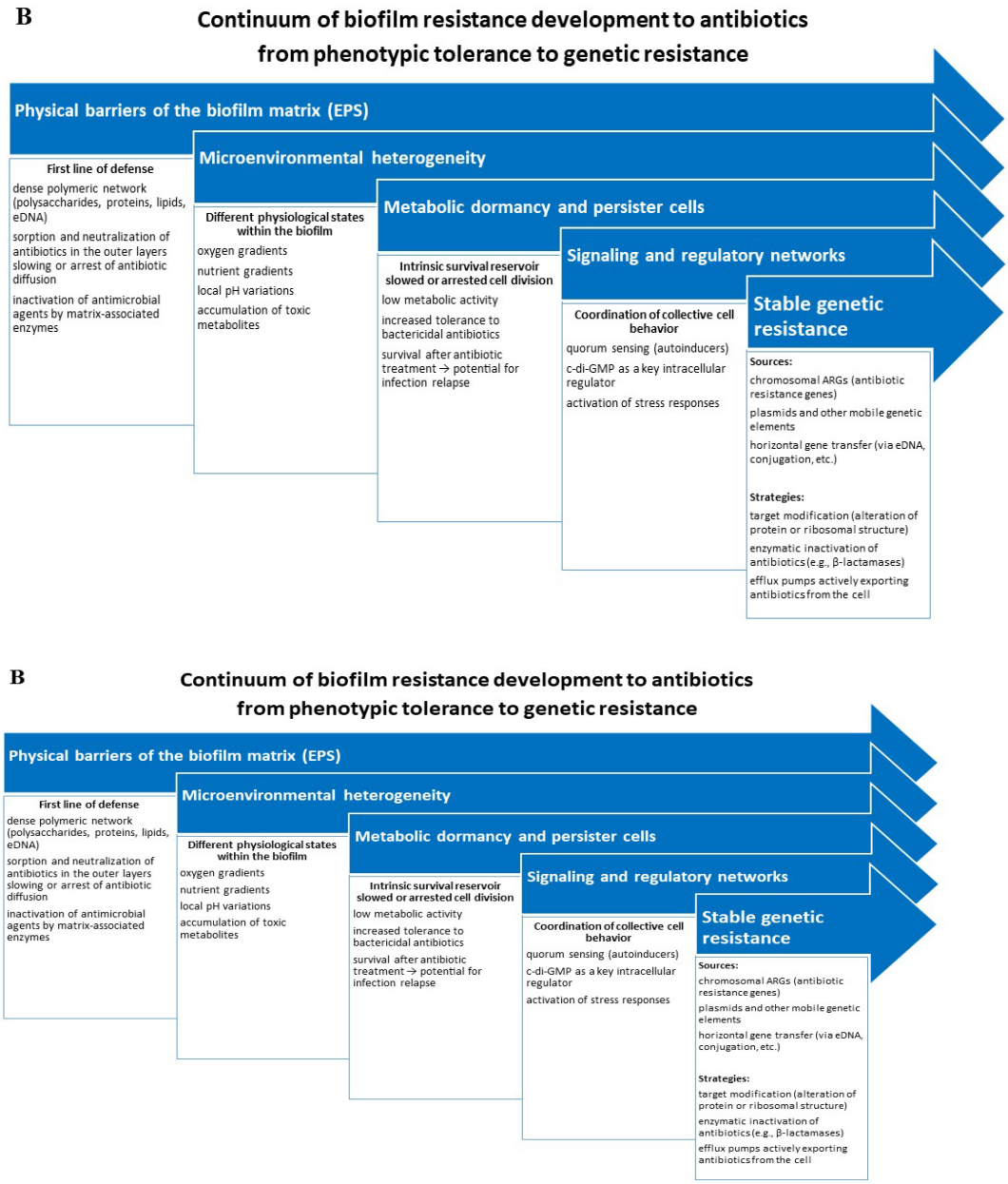
As noted by Shree P. et al., the effectiveness of an antibiotic against microorganisms in a biofilm is largely determined by the extent of its penetration into the matrix. This process depends on the structural features of the biofilm, species- and strain-specific bacterial characteristics, and the physicochemical properties of the drug (Cangui-Panchi, 2023).

Panlilio H., and Rice C.V. showed that eDNA and the exopolysaccharide component of the EPS matrix are important contributors to the development of antibiotic resistance. EPS is a key structural element that ensures architectural stability of the biofilm and participates in its adaptive response to stress factors, including antibiotic exposure (Panlilio, 2021). Owing to its high viscosity and polymeric nature, the EPS matrix creates a selective barrier that slows antibiotic penetration and alters its local concentration within the biofilm, thereby increasing the likelihood of antibiotic inactivation by matrix-associated enzymes (Panlilio, 2021).

The nature of antimicrobial penetration is also influenced by the morphological features of the biofilm, including its thickness, density, degree of heterogeneity, and presence of microchannels. These parameters determine diffusion efficiency: in denser or thicker regions, movement of antibiotic molecules is substantially slowed, reducing access to the deeper cellular layers. As a result, antimicrobial agents may be retained in peripheral regions of the biofilm, where they are partially neutralized by EPS-associated enzymes, while the inner cellular layers remain inaccessible (Cruz, 2021; Pinto, 2020).

The heterogeneity of the bacterial population within biofilms results in the formation of metabolically distinct microcolonies. According to the zonal model, each cell responds to local microenvironmental conditions, leading to the emergence of diverse physiological states even within a single biofilm (Kirketerp-Møller, 2020). These differences are associated with changes in pH, hydrogen peroxide concentration, the presence of noncellular components, and nutrient deficiency (Flemming, 2016). The deepest layers of the biofilm often experience pronounced starvation due to limited diffusion and intensive substrate consumption by peripheral cells, which promotes accumulation of persister or “dormant” cells with slowed metabolism and reduced susceptibility to antibiotics (Upadhyay, 2025; Ciofu, 2023). These zones have been identified as foci of increased microbial resistance (Liu, 2022; Liu, 2022).

Figure 2 (A, B) presents the key spatial and functional aspects of biofilm antibiotic resistance.



B
Continuum of biofilm resistance development to antibiotics
from phenotypic tolerance to genetic resistance
Physical barriers of the biofilm matrix (EPS)
First line of defense
dense polymeric network (polysaccharides, proteins, lipids, eDNA)
sorption and neutralization of antibiotics in the outer layers slowing or arrest of antibiotic diffusion
inactivation of antimicrobial agents by matrix-associated enzymes
Microenvironmental heterogeneity
Different physiological states within the biofilm
oxygen gradients
nutrient gradients
local pH variations
accumulation of toxic metabolites
Metabolic dormancy and persister cells
Intrinsic survival reservoir slowed or arrested cell division
low metabolic activity
increased tolerance to bactericidal antibiotics
survival after antibiotic treatment → potential for infection relapse
Signaling and regulatory networks
Coordination of collective cell behavior
quorum sensing (autoinducers)
c-di-GMP as a key intracellular regulator
activation of stress responses
Stable genetic resistance
Sources:
chromosomal ARGs (antibiotic resistance genes)
plasmids and other mobile genetic elements
horizontal gene transfer (via eDNA, conjugation, etc.)
Strategies:
target modification (alteration of protein or ribosomal structure)
enzymatic inactivation of antibiotics (e.g., β-lactamases)
efflux pumps actively exporting antibiotics from the cell

Fig. 2. Mechanisms underlying antibiotic tolerance of microbial biofilms

A. Structural organization of a biofilm and factors affecting antibiotic penetration, including diffusion limitation, metabolic heterogeneity, and formation of dormant persister cells.

B. Major structural, physiological, regulatory, and genetic mechanisms contributing to biofilm antibiotic tolerance and resistance.

Thus, the internal structural organization of the biofilm, together with physical, metabolic, and genetic factors, forms diverse genotypic and phenotypic variants that ensure unique strategies of survival and drug resistance (Fig. 2 A, B).

Therefore, biofilm resistance to antibiotics is the result of interaction among several levels of defense: the physical barrier of the extracellular matrix, metabolic inertness and formation of persister cells, heterogeneity of the microenvironment, and dissemination of antibiotic resistance genes. The synergism of these mechanisms creates major difficulties in the treatment of biofilm infections and promotes their progression to chronic forms. The complex nature of biofilm resistance, in turn, necessitates experimental models that reproduce their architecture and allow adequate assessment of antibiotic susceptibility under conditions that closely mimic the natural environment.

The following section is devoted to this issue and addresses current approaches to cultivation and investigation of bacterial biofilms.

Methodological problem of assessing antibiotic susceptibility in biofilm-associated infections. The effectiveness of antibiotic therapy is usually determined on the basis of minimal inhibitory concentrations (MICs) for planktonic bacteria. However, these concentrations are too low for microorganisms protected by biofilms, and, as a consequence, patients may suffer from persistent infection for weeks or months, often with recurrent, more aggressive exacerbations (Thakre, 2024). Many clinical studies have established that patients with infections caused by biofilm-forming bacteria require higher antibiotic doses and longer treatment courses than those used for infections caused by planktonic bacteria (da Silva, 2021; Pires, 2022). Therefore, theoretically, treatment efficacy in biofilm-associated infections should be higher when antibiotics are selected with regard to biofilm susceptibility than when therapeutic regimens are based on planktonic susceptibility testing.

However, the evidence base regarding the clinical usefulness of selecting antibiotic therapy on the basis of biofilm susceptibility testing remains insufficient, and the available data do not confirm a substantial advantage of this approach over the traditional assessment of susceptibility of planktonic forms.

Methods for biofilm cultivation and determination of their antibiotic susceptibility. Unlike methods for studying planktonic cells, there are currently no universal standardized methods for assessing biofilm susceptibility to antimicrobial agents. This is explained by the considerable diversity of laboratory models used to simulate biofilms formation, as well as differences among them in terms of growth conditions, biofilm architecture, and analytical endpoints. Each method has its own advantages and limitations, and the choice of approach is largely determined by the aim of the study, from qualitative confirmation of biofilm-forming ability to quantitative comparison of the susceptibility of biofilm and planktonic forms to antibiotics (Upadhyay, 2025; Ciofu, 2023; Su, 2022; Han, 2023).

Among the earliest methods were simple static qualitative models. The tube method for biofilm detection described by Christensen et al. was based on the ability of cells to adhere to the walls of a tube and form a visible film, which was detected by crystal violet staining followed by visual assessment of staining intensity (Christensen, 1982). This approach was simple to perform, required no specialized equipment, and allowed rapid classification of strains as weak, moderate, or strong biofilm producers. At the same time, it remained sufficiently subjective, poorly reproducible, and of limited utility for analysis of large numbers of isolates or subtle differences among them.

A similar role was played by the use of Congo Red Agar (CRA), proposed by Freeman et al. (1989). On this medium, biofilm-forming strains produce black, dry crystalline colonies as a result of interaction of Congo red with exopolysaccharides. Thus, the CRA test reflects the ability to produce matrix and indirectly, biofilm formation. Its advantages include low cost, simplicity, and the possibility of rapid primary screening of numerous isolates; disadvantages include its qualitative nature, dependence of the result on medium composition, and the fact that not all bacterial species form typical “black” colonies (Freeman, 1989).

The gold standard for biofilm cultivation is considered to be the quantitative microtiter plate biofilm assay described by G.D. Christensen et al. (1985) (Christensen, 1985) and further detailed by Stepanovic et al. (2000) (Stepanovic, 2000). In this model, the biofilm forms on the walls and bottom of polystyrene microplate wells, after which planktonic cells are washed away, the

adherent biomass is stained with crystal violet, and quantitatively assessed by optical density (OD) at a specified wavelength. The threshold optical density value (ODc) is calculated as the mean OD of the negative control plus three standard deviations, and the degree of biofilm formation is interpreted based on the ratio of the experimental sample OD to ODc (Han, 2023; Christensen, 1982; Stepanovic, 2000).

This approach made it possible to transform assessment of biofilm formation intensity into a standardized quantitative test suitable for comparing strains, growth conditions, and antibiotic effects. The high throughput of the 96-well format made this method a convenient tool for genetic screening, testing of different nutrient media and induction factors, and investigation of early adhesion stages in a broad range of microorganisms, including *Pseudomonas aeruginosa, Vibrio cholerae, Escherichia coli*, staphylococci, enterococci, mycobacteria, and fungi. The main limitation is that static conditions do not reproduce shear forces and *in vivo* hydrodynamics, and the resulting biofilms usually do not reach a high degree of maturity (Christensen, 1982; Freeman, 1989).

In order to approximate natural and clinical conditions, dynamic biofilm models were developed in flow systems, drip-flow reactors, tubular reactors, coupon-based rotating disk reactors, and similar systems (Ciofu, 2023).

An important milestone was the development of standardized protocols approved by the American Society for Testing and Materials (ASTM, www.astm.org). ASTM standards describe the growth of biofilms, particularly *P. aeruginosa*, in a continuous-flow drip reactor (ASTM E2647), a rotating disk reactor (ASTM E2196), and a CDC biofilm reactor (ASTM E2562), in which shear force levels and flow parameters can be controlled. These systems allow formation of mature biofilms on various materials, assessment of the effects of disinfectants and antibiotics under flow conditions, and combination of quantitative viable cell determination with microscopic analysis of architecture. Their advantages include high ecological validity and the possibility of modeling the environment of medical implants or pipelines; disadvantages include equipment complexity, low throughput, and the fact that current standards are focused mainly on *P. aeruginosa* (Kher, 2023).

Regardless of whether static or dynamic models are used, the classical quantitative approach to evaluating biofilm susceptibility to antimicrobial agents remains colony-forming unit (CFU) counting. After antibiotic exposure, the biofilm mass is removed from the surface (coupons, glass coverslips, silicone tubing, membranes, etc.) by mechanical methods, resuspended, serially diluted, and plated on agar to calculate the logarithmic reduction in the number of viable cells (Wannigama, 2020). This approach is sensitive and universal, but labor-intensive, especially when it is necessary to analyze a wide concentration range, several strains, or complex biofilm growth systems; an additional problem is incomplete recovery of cells from the substrate.

The development of high-throughput technologies contributed to the introduction of the Calgary Biofilm Device, which formed the basis of the MBEC (minimum biofilm eradication concentration) assay and commercial tests by Innovotech Inc., including bioFILM PA, InnovoSCEPT Human Gram Positive, and InnovoSCEPT Human Gram Negative, which received regulatory approval for clinical use (Ceri, 2001; Ceri, 1999; Harrison, 2010). In these systems, biofilms are grown on pegs attached to the lid of a 96-well microplate. After biofilm formation, the lid is transferred to a plate containing a series of antibiotic dilutions and then to a plate with fresh medium for recovery; subsequent ultrasound treatment enables detachment of cells from peg surfaces and assessment of their viability (Kher, 2023). This format makes it possible to simultaneously determine MIC, minimum bactericidal concentration for biofilm cells (MBC-B), and MBEC, compare the susceptibility of biofilm and planktonic forms, and adapt peg composition to model different surface types. The advantages of the MBEC assay include a standardized format, high throughput, and clinical relevance; its limitations are relatively high cost and the need for specialized equipment (Pires, 2022; Mah, 2014).

Another direction has been the use of metabolic indicators of viability. The technology described by Wannigama D.L. et al. is based on the use of PrestoBlue (resazurin), which is reduced by metabolically active cells, thereby changing optical density or fluorescence. By determining the signal after treatment of biofilms with different antibiotic concentrations, MBEC can be assessed and drug effectiveness compared (Wannigama, 2019; Wannigama, 2020). The advantage of this approach is avoidance of labor-intensive colony counting and suitability for high-

throughput formats; its limitation lies in the dependence of the result on the metabolic state of the cells, which may lead to underestimation of persister populations.

To assess specifically the bactericidal effect, the MBC-B test was proposed, in which biofilms are formed directly on the walls of 96-well microplates, exposed to a gradient of antibiotic concentrations, and then the detached cells are transferred to nutrient medium to determine the concentration that completely suppresses growth (Ceri, 1999).

Table 1 summarizes the key characteristics of the most common models used to obtain and study biofilms and their antibiotic resistance.

Thus, the current arsenal of methods for biofilm research ranges from simple qualitative tests to complex dynamic systems and high-throughput platforms that make it possible to assess MIC, MBC-B, and MBEC for biofilm populations. Static models enable rapid screening of strains and factors, whereas flow reactors and ASTM standards approximate real natural, clinical, and technogenic conditions. High-throughput systems such as MBEC, metabolic assays using PrestoBlue, and MBC-B tests create a bridge between fundamental research and the needs of clinical practice.

Table 1

Comparative characteristics of laboratory methods for biofilm generation

Method	Type (static/ dynamic)	Principle of the method / where the biofilm forms	What it measures / what it allows one to assess	Advantages	Disadvantages	Typical areas of application
1	2	3	4	5	6	7
Tube Method (TM)	Static	Biofilm forms on tube walls; assessment after crystal violet staining	Presence of biofilm, semi-quantitative staining intensity	Simple, inexpensive, requires no equipment	Low reproducibility, subjective assessment, low sensitivity	Screening of strains for biofilm-forming ability
Tissue Culture Plate (TCP), 96-well plate	Static	Biofilm forms in plate wells; quantitative assessment by OD after staining	Degree of biofilm formation; strain comparison; antibiotic effects	High throughput; quantitative analysis; small volume	Does not model mature biofilms; antibiotic diffusion is inadequate compared with real conditions	Genetic studies, primary screening of biofilm formation, inhibitor testing
Congo Red Agar (CRA)	Static	Colonies become black/dark upon polysaccharide production	Ability to produce EPS, indirectly biofilm formation	Very simple; rapid; requires no specialized equipment	Low specificity; not all strains produce pigments; subjectivity	Initial qualitative screening of matrix production
Colony biofilms (agar biofilms)	Static	Biofilm forms on the agar surface; allows observation of structural morphology	Colony architecture, diffusion gradients	Possibility of visualization; suitable for microscopy	Limited model: does not reproduce shear forces	Study of biofilm differentiation and morphology

1	2	3	4	5	6	7
Flow Cell Systems	Dynamic	Biofilm forms on the chamber surface under constant medium flow	Real-time growth, architecture, effects of flow and antimicro-bials	Closest to natural conditions; live microscopy possible (confocal)	Expensive equipment, operational complexity, low throughput	Fundamental studies of biofilm architecture, growth/disrupti on dynamics
Drip Flow Reactor (ASTM E2647)	Dynamic	Biofilm formation under drip flow on a surface under low shear	Mature biofilms, effects of antibiotics under flow conditions	Standardized method; high reproducibi-lity	Requires a specialized reactor; more expensive	Evaluation of disinfectants, antibiotic testing for mature biofilms
Rotating Disk Reactor (ASTM E2196)	Dynamic	Biofilm grows on disks under controlled shear forces	Effect of shear stress; mature biofilms	Good simulation of tubular systems	High structural complexity	Study of industrial and medical biofilms
CDC Biofilm Reactor (ASTM E2562)	Dynamic	Biofilm forms on standard coupons in a stirred reactor	Number of viable cells; antibiotic effective-ness	High reproducibilit y; used in ASTM standards	Low throughput, cost	Industrial microbiology, disinfectant testing
Calgary Biofilm Device / MBEC Assay	Semi-dynamic	Biofilms grow on pegs attached to the lid of a 96-well plate	MBEC, MIC, MBC; comparative resistance of biofilms and planktonic cells	High throughput; standardizati on; quantitative nature	More expensive; not all species form biofilms well on pegs	Antimicrobial studies, pharmaceutica l testing
PrestoBlue / metabolic viability assays	Static or dynamic (depen-ding on model)	Assessment of cell activity through resazurin reduction	Biofilm cell viability, MBEC	Rapid, sensitive, suitable for comparisons	Result depends on metabolism rather than cell number	High-throughput antibiotic screening

Discussion. The data summarized in this review confirm that biofilm-associated resistance to antibacterial agents cannot be attributed to a single mechanism. It results from the combined action of structural, physiological, regulatory, and genetic factors. The extracellular matrix limits antimicrobial penetration, modifies local drug concentrations, and creates conditions for partial antibiotic inactivation. At the same time, the spatial heterogeneity of the biofilm leads to the formation of zones with different levels of oxygen, nutrients, metabolites, and signaling molecules. Under these conditions, part of the bacterial population shifts toward reduced metabolic activity or persistence, which substantially decreases the efficacy of antibiotics targeting actively dividing cells.

The distinction between genetically determined resistance and phenotypic tolerance of biofilm-embedded cells is particularly important in the clinical context. Standard antimicrobial susceptibility testing based on planktonic bacteria does not always reflect the actual ability of an antibiotic to suppress or eradicate a biofilm population. Minimal inhibitory concentrations determined for planktonic cells may be insufficient for biofilms, where diffusion barriers, enzymatic inactivation, efflux systems, target modification, horizontal transfer of resistance genes, and persister cell formation act simultaneously. This explains why biofilm-associated infections often follow a prolonged, recurrent, or chronic course.

Comparison of laboratory models shows that none of the currently available approaches is universally applicable to all research and clinical tasks. Static models, including the tube method, Congo Red Agar, and microtiter plate assays, remain useful for primary screening of biofilm-forming ability, biomass comparison, and assessment of individual environmental or antimicrobial factors. Their main advantages are simplicity, accessibility, and high throughput. However, these systems reproduce the spatial organization of mature biofilms, hydrodynamic conditions, shear forces, and nutrient gradients of natural, industrial, and clinical environments only to a limited extent.

Dynamic systems, including flow-cell models, drip-flow reactors, rotating disk reactors, and CDC biofilm reactors, more closely reproduce the conditions under which mature biofilms are formed. They allow investigation of the effects of flow, shear stress, surface properties, cultivation time, and nutrient supply. These models have higher biological relevance, especially for studying biofilms on medical devices, in water systems, pipelines, and other surface-associated ecosystems. However, their broader application is limited by equipment costs, lower throughput, technical complexity, and the need for specialized personnel training.

Methods used to assess the antimicrobial susceptibility of biofilm cells also differ in their analytical value. Colony-forming unit counting remains one of the most universal approaches for determining the number of viable cells after antimicrobial exposure. Nevertheless, it is labor-intensive and depends on the completeness of cell detachment from the surface. MBEC-based approaches and the Calgary Biofilm Device provide a more standardized platform for comparing antibiotic activity against planktonic and biofilm forms, whereas MBC-B specifically evaluates the bactericidal effect against biofilm populations. Metabolic viability assays are suitable for rapid screening, but their results should be interpreted with caution because the metabolic heterogeneity of biofilms may lead to underestimation of low-activity or persister cells.

Therefore, the choice of a model should be determined not only by method availability but also by the specific aim of the study. Simple static systems are appropriate for primary screening. Dynamic models are more suitable for investigating biofilm architecture, maturation, and disruption. For evaluating antibiotics, disinfectants, or potential antibiofilm agents, the most informative strategy is the combination of several analytical approaches, including viable cell counting, MBEC or MBC-B determination, metabolic assays, and, where possible, microscopic visualization. Such a combined approach makes it possible to assess not only biomass reduction or cell viability but also changes in biofilm structure, metabolic activity, and recovery potential.

An important methodological implication of this review is the need to clearly distinguish between biofilm resistance, tolerance, and persistence. The interchangeable use of these terms may lead to incorrect interpretation of experimental data and overestimation of the predictive value of individual testing methods. For practical application, laboratory models should not only demonstrate differences between planktonic and biofilm-associated cells but also help predict the potential efficacy of therapeutic or disinfectant interventions under conditions close to clinical or industrial settings.

Thus, further development of biofilm research should focus on standardization of experimental conditions, selection of relevant endpoints, harmonization of interpretation criteria, and integration of reproducibility with biological relevance. This is important not only for a deeper understanding of the biofilm mode of microbial life but also for the development of more effective approaches to the prevention, diagnosis, and treatment of biofilm-associated infections.

Conclusions

1. Biofilms are highly organized microbial communities whose resistance to antibacterial agents results from the interaction of the extracellular matrix, spatial and metabolic heterogeneity, persister cells, regulatory mechanisms, and genetically determined resistance.
2. Standard susceptibility testing of planktonic microorganisms does not fully reflect the behavior of biofilm populations. Therefore, specialized models and endpoints, including MBEC, MBC-B, viable cell counting, and metabolic viability assays, are required for studying biofilm-associated infections.
3. Static models are useful for primary screening and quantitative biomass assessment, whereas dynamic systems better reproduce the conditions of mature biofilm formation and allow analysis of flow, surface properties, and shear stress.

4. Further development of standardized, reproducible, and biologically relevant methods for biofilm investigation is essential for improving laboratory assessment of antimicrobial activity and for designing more effective strategies to control biofilm-associated infections.

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Біоплівки мікроорганізмів: молекулярні механізми стійкості до антибіотиків та методи їх дослідження

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Резюме. *Узагальнення сучасних даних про молекулярні механізми стійкості біоплівок до антибактеріальних засобів та аналіз лабораторних підходів, що застосовуються для культивування, візуалізації й визначення чутливості біоплівок до антимікробних препаратів. Проведено аналітичний огляд наукових публікацій українських і зарубіжних авторів, присвячених біоплівкам, антибіотикорезистентності та методам їх дослідження. Для добору джерел використано відкриті наукометричні та бібліографічні ресурси, зокрема PubMed, Google Scholar, ScienceDirect, SpringerLink, Scopus, а також вітчизняні бібліотечні фонди. Пошук здійснювали за ключовими словами, що охоплювали біоплівки, антимікробну резистентність, антибіотикотолерантність, молекулярні механізми стійкості, методи вивчення біоплівок і біоплівко-асоційовані інфекції. Показано, що резистентність біоплівок має багатофакторний характер і формується внаслідок поєднаної дії кількох механізмів. Вирішальне значення мають бар'єрні властивості позаклітинного матриксу, до складу якого входять екзополісахариди, білки, ліпіди та позаклітинна ДНК, а також виражена просторово-метаболічна гетерогенність клітин усередині біоплівки. Обмежена дифузія антибіотиків у глибокі шари, поява персистентних клітин, зниження темпів росту, активація ефлюксних систем, ферментативна інактивація препаратів, зміна антибактеріальних мішеней і горизонтальне поширення генів резистентності разом забезпечують підвищену толерантність мікроорганізмів до терапії. У роботі також узагальнено сучасні підходи до дослідження біоплівок. Статичні моделі, зокрема пробірковий метод, Congo Red Agar і планшетний тест у 96-лункових мікропланшетах, придатні для первинного скринінгу та кількісної оцінки біомаси, але лише частково відтворюють умови природних і клінічних систем. Динамічні моделі, включно з flow-cell системами, drip-flow, rotating disk та CDC-реакторами, дають змогу моделювати зрілі біоплівки за контрольованого потоку й зсувних навантажень, однак потребують складнішого обладнання. Окрему групу становлять методи оцінки чутливості біоплівкових клітин, зокрема визначення MBEC, MBC-B, підрахунок колонієутворювальних одиниць і метаболічні тести на основі індикаторів життєздатності, які дозволяють точніше порівнювати ефективність антибіотиків щодо планктонних і біоплівкових форм. Стійкість біоплівок до антибактеріальних препаратів не може бути пояснена одним фактором, оскільки вона є наслідком структурної організації біоплівки, фізіологічної неоднорідності клітинної популяції та реалізації генетично й фенотипово зумовлених механізмів захисту. Наявні лабораторні моделі істотно розширили можливості дослідження біоплівок, проте жодна з них не є універсальною. Подальший розвиток відтворюваних і стандартизованих підходів, які поєднуватимуть біологічну релевантність із аналітичною надійністю, є необхідною передумовою для вдосконалення діагностики та підвищення ефективності лікування біоплівко-асоційованих інфекцій.*

Ключові слова: біоплівки; антибіотикорезистентність; антибіотикотолерантність; позаклітинний матрикс; персистентні клітини; MBEC; MBC-B; методи дослідження біоплівок
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