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ENVIRONMENTAL MICROBIOLOGY - REVIEW

The Determination, Monitoring, Molecular Mechanisms and Formation of Biofilm in *E. coli*

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Abstract

Biofilms are cell assemblies embedded in an exopolysaccharide matrix formed by microorganisms of a single or many different species. This matrix in which they are embedded protects the bacteria from external influences and antimicrobial effects. The biofilm structure that microorganisms form to protect themselves from harsh environmental conditions and survive is found in nature in many different environments. These environments where biofilm formation occurs have in common that they are in contact with fluids. The gene expression of bacteria in complex biofilm differs from that of bacteria in the planktonic state. The differences in biofilm cell expression are one of the effects of community life. Means of quorum sensing, bacteria can act in coordination with each other. At the same time, while biofilm formation provides many benefits to bacteria, it has positive and negative effects in many different areas. Depending on where they occur, biofilms can cause serious health problems, contamination risks, corrosion, and heat and efficiency losses. However, they can also be used in water treatment plants, bioremediation, and energy production with microbial fuel cells. In this review, the basic steps of biofilm formation and biofilm regulation in the model organism *Escherichia coli* were discussed. Finally, the methods by which biofilm formation can be detected and monitored were briefly discussed.

Keywords *Escherichia coli* · Biofilm Formation · Bacteria · Biofilm Imaging · Biofilm Detection Methods

Introduction

Bacteria develop different strategies to survive under stressful conditions in the natural environment. Sporulation, dormancy, and biofilm formation are the most important of these strategies [1–7]. Biofilms are microbial communities associated with biotic or abiotic surfaces and embedded in a self-produced polymeric matrix [8, 9]. Biofilms are not just cells embedded in a polymeric matrix. The bacteria in these structures can be described as multicellular life because they coordinate with each other, respond together to internal and external signals, and control their density [10]. The matrix is combined with extracellular polymeric substances and the organisms in the population can be homogeneous or heterogeneous. Organisms that reside in the matrix take advantage of the protection provided by the biofilm. Bacteria in biofilms are more resistant and stable to phagocytosis, nutrient starvation, pH changes, biocides, and antimicrobials compared to free-floating bacteria [8, 9].

They are easily found in many environments and play a major role in industrial activities [11]. The formation of biofilms can cause food spoilage and equipment damage, leading to serious hygienic and economic problems in the dairy industry [12]. The persistence of some foodborne pathogens contained in biofilms formed on food contact surfaces affects the quality, quantity, and safety of food. As an example from medicine, biofilms are found on catheters, prosthetic heart valves, contact lenses, and various implant surfaces. In such cases, removal of the implant is generally the only option for treatment, but it affects the health of the patient and causes higher costs [11]. Organisms that reside in biofilms play a role in catalyzing chemical and biological reactions that occur in pipes and tanks, causing corrosion of metals. The thickening of biofilms in plate heat exchangers and inside

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piping results in a significant reduction in heat transfer efficiency, forcing facilities to spend more money [13].

They may have many negative effects, but in the end they have a great part in the ecology and sustainability of nature. It has many beneficial aspects, such as treating wastewater, protecting and promoting plant growth, eliminating oil and gasoline spills (bioremediation), extracting precious metals such as gold, and preventing and combating corrosion [14].

Biofilm Formation Stages

As shown in Figure 1, biofilm formation consists of three main steps: attachment, maturation, and dispersion. The attachment phase occurs in two steps. In the first step, attachment occurs, the adhesion of the cells to the surface is weak and they can detach from the surface. In the second step of attachment, the bacteria that are not detached from the surface contribute to the early structuring of the biofilm. This step is not reversible, and microcolonies are also formed. Microcolonies develop and become a mature form. When conditions change, such as a decrease in envorimental temperature, nutrients, and oxygen, they convert back to planktonic forms, resulting in biofilm dissolution [15–17].

Attachment

The attachment step can be examined as two phases: reversible and irreversible. When the biofilm is in the irreversible attachment phase, it can resist stronger chemical or physical shear forces.

Reversible Attachment

When suitable nutrient conditions exist in the environment, biofilm can develop. The attachment surfaces of biofilm-forming cells can be abiotic, such as glass [18], plastic [19], metal [20], stainless steel [21], medical prostheses and implants [22], or biotic, such as, human skin [23], animal tissues [24] and epithelial cells [25]. Examples of factors that prevent biofilm formation include environmental pH [26], ionic strength [27], environmental factors [28], and hydrodynamic and electrostatic forces in liquid media [29]. At the same time, the components of the bacterial surface are another factor affecting the distance or convergence of bacteria from the surface [15]. In order to adhere to the surface, the microorganisms must overcome these repulsive forces [17]. Physical forces such as the stickiness of exopolysaccharides (EPS), hydrophobic interactions, and van der Waals forces ensure reversible attachment of microorganisms to the surface [30, 31]. Flagella, a whip-like filamentous appendage that provides locomotion for bacteria, can overcome the repulsive forces that may prevent cell-surface interaction and allow cells to adhere to surfaces [32]. Kostakioti et al. (2013) found that bacterial flagella are necessary to overcome the hydrodynamic and repulsive forces between the bacteria and the surface [15]. Flagellar motility is essential for initial attachment and has been demonstrated in pathogens such as *Vibrio cholerae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Escherichia coli* [33–38]. Flagella mutant strains of *L. monocytogenes* disrupt their surface adhesion when incubated for a short time. However, mutant cells

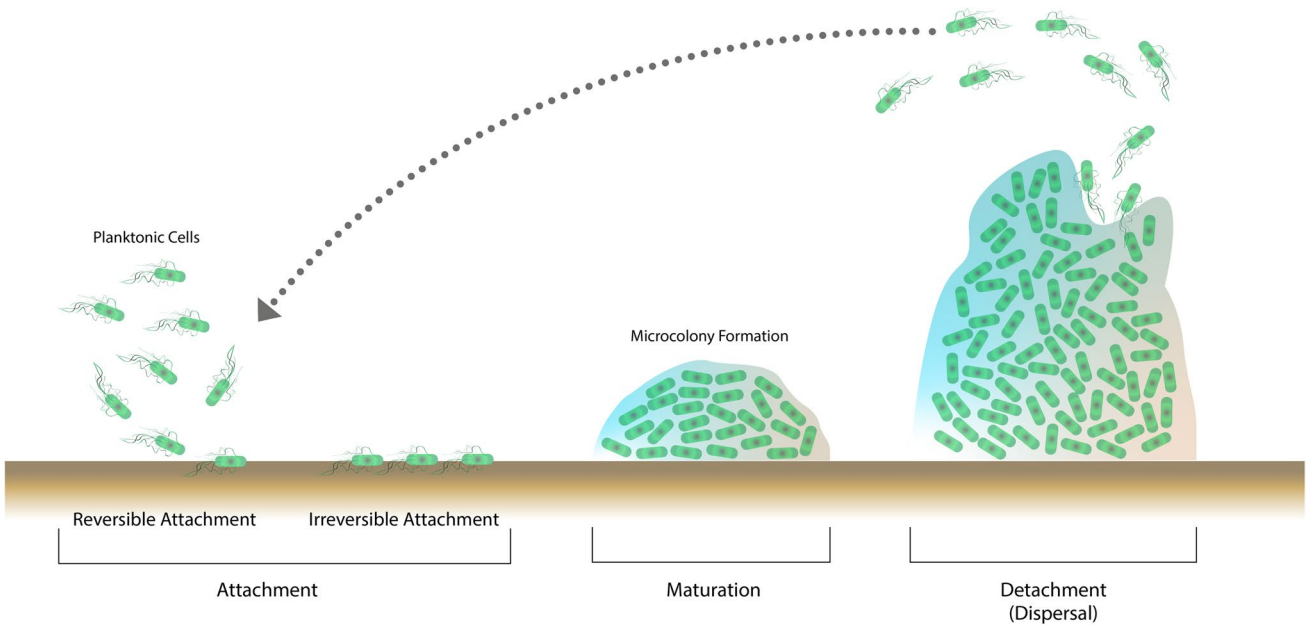

Figure 1 Biofilm Formation Stages

without flagella achieve almost the same degree of surface coverage as cells with flagella upon prolonged incubation. This indicates how important flagella are in the initial and early attachment phase [39]. As shown in the study of *L. monocytogenes*, flagella-mediated movement is an important factor in adhesion, but even bacteria without flagella can adhere to surfaces with expression of robust adhesion factors [17]. Flagella serve to actively move *E. coli* and increase the interaction between bacteria and the surface, providing the initial cell-surface contact for adhesion [40]. However, "initial attachment" is dynamic and reversible, so bacteria become planktonic in response to hydrodynamic forces, repulsive forces, or the presence of nutrients [41–43].

Irreversible Attachment

Irreversible attachment involves bacterial surface components and adhesins in addition to dipole-dipole interactions, hydrophobic interactions, hydrogen, ionic, and covalent bonds [44, 45]. Bacterial cells are generally negatively charged and therefore have a better chance of overcoming repulsive forces and settling on positively charged surfaces. Bacterial surface constituents, another factor in colonization, vary from bacterium to bacterium [44]. Gram-negative bacteria have lipopolysaccharides (LPS) in their outer membrane structure. LPS are divided into three parts: O-antigen, lipid A, and core oligosaccharide. Teichoic acid (TA), another element of the cell wall structure, is found in Gram-positive bacteria. The loads created by these structures on the outer surfaces of the bacteria enable the bacteria to adhere to the surfaces, thus forming a biofilm [46]. There are many adhesins that extend from the cell surface to the outside of the cell. Bacterial adhesins play a role in biofilm formation. Examples include flagella, pili/fimbriae, and non-fimbrial adhesins [47]. These surface organells mediate the first physical contact between substrates and bacterial cells occurs [47–49]. Pili/fimbriae are filamentous cell-surface adhesins that allow bacteria to adhere to each other and form initial cell-surface attachment [50, 51]. Pili play an active role in the initial attachment of *Klebsiella pneumoniae*, *Clostridium difficile*, *Acinetobacter baumannii* and *Streptococcus agalactiae* to different surfaces [50–52]. Curli fimbriae, antigen 43, and type 1 fimbriae in *E. coli* have been found to increase initial surface adhesion and to be involved in irreversible attachment [53–55]. In addition, several adhesins mediate permanent surface adhesion in some bacteria. The Holdfast polysaccharide mediates the formation of the first layer in *Caulobacter crescentus*. The intercellular polysaccharide adhesins (PIA) are essential for biofilm formation and provide cell-to-cell adhesion in *Staphylococcus epidermidis* [56].

The quorum sensing (QS) mechanism initiate biofilm formation by providing a cell-to-cell signaling [57]. Taking advantage of the QS, bacteria synthesize and release the initial messengers, such as chemical signals, that enable cell-to-cell communication [58, 59]. Cell-to-cell signaling mechanisms are used in the regulation of biofilm formation in Gram-negative and Gram-positive bacteria. Gram-negative bacteria use acyl homoserine lactones (AHLs) as cellular signals, whereas Gram-positive bacteria use oligopeptides. Oligopeptides are referred to as universal autoinducers (AIs) because they can be used in both negative and positive Gram reactive bacteria [14].

EPS production is triggered by the QS mechanism in adherent bacteria, providing irreversible attachment. EPS is secreted extracellularly after synthesis by bacteria and is an important component of the extracellular matrix. EPS secreted by the cell ensures the attachment of biofilms to surfaces and the cohesion of bacteria. In doing so, it utilizes ion-bridging and hydrophobic interactions [60, 61]. In general, EPS plays a crucial role in genetic modifications, adhesion to surfaces, biofilm formation and structure, signal transduction, cell protection, cell-cell recognition, and water binding [14]. The main components of EPS, mainly polysaccharides, proteins, DNA, lipids, and other polymeric compounds, vary according to environmental conditions and bacterial species [14]. Polysaccharides, an important component of the EPS matrix, are important for biofilm maturation and growth of many bacteria [62].

Biofilm Maturation

After irreversible attachment, the attached bacterial cells proliferate. In direct proportion to the increase in cell number, EPS production increases, and thus microcolony formation occurs [63]. Simultaneously with the formation of the microcolony, the expression of many genes involved in the formation of the extracellular matrix is also triggered, and the factors involved in biofilm formation are regulated [15, 63]. With the regulation of the factors, the formation of the extracellular matrix occurs and then the three-dimensional structure of the biofilm is formed [17]. Matrix formation is followed by the formation of water-filled channels that act as a circulatory system, transporting nutrients to cell communities and removing waste products [64]. After the first layer of biofilm structure is formed, other bacteria of the same species and bacteria of other species in the environment are also incorporated into the biofilm structure. In this way, the biofilm structure develops from a thin layer to a "mushroom" or "tower" shaped structure [16].

The biofilm is a dynamic structure that changes according to environmental conditions. For example, *P. aeruginosa*, which is used as a model in biofilm studies, forms macrocolonies with rod-shaped cells in the presence of oxygen, and the channels formed between these macrocolonies form a mushroom-shaped structure. On the other hand, under

anoxic conditions, *P. aeruginosa* forms macrocolonies with long filamentous cells, and the channels between these macrocolonies form a three-dimensional network-like structure [65, 66]. These morphological changes in the biofilm alter the distribution of substances within the biofilm structure, providing a balance of waste products and nutrients. This contributes to the metabolic adaptation of cells to oxic or anoxic conditions [65].

After a dense biofilm is formed, bacteria cause gradients according to their existing metabolic structures and oxygen tolerance. This leads, for example, to the presence of anaerobic bacteria in the deeper layers of the biofilm where oxygen is not available for their growth. As the biofilm structure matures, the amounts of biofilm structural elements such as polysaccharides, proteins and DNA in the biofilm are increased by the cells in the biofilm [16].

Biofilm Dispersion

In this final stage of biofilm formation, bacteria separate from the mature biofilm and transition to planktonic form to form new biofilms in other regions [17]. This stage is the result of physiological and environmental conditions that explain the formation of biofilms by combining bacteria with new substrates. In this stage, which is a natural process of biofilm, bacteria can form microcolonies on new surfaces by leaving the biofilm through certain physiological or environmental signals [67]. Numerous factors affect the spread of biofilms, such as nutrient starvation, intense competition, degradation of the biofilm matrix and quorum sensing by enzymes, oxygen starvation, and an overly large population [68]. Environmental signals, signal transduction pathways, and effectors involved in biofilm dispersal make the process complex [69]. Dispersion can occur throughout the biofilm formation or only in a part of it [16].

The dispersion mechanism in bacteria occurs in three general phases: First, the cells leave the microcolonies, the cell moves to a new substrate, and the cell adheres to the new substrate, initiating a new biofilm formation process [69, 70]. Detachment can occur actively in response to changes in the environment surrounding cells in biofilms, such as antimicrobial stress, enzymes that degrade the structure of the matrix, and nutrient starvation, or it can occur passively under the influence of external factors such as shear forces [69, 71, 72]. During active dispersion, the expression of genes related to cell motility, such as flagellar synthesis and EPS degradation, is upregulated, whereas the expression of genes involved in EPS production, attachment, and fimbrial synthesis is generally downregulated [15]. The best known example of active detachment is seeding dispersal, which occurs when clumps of cells or planktonic cells are rapidly released from the center of the biofilm. Following this detachment, cavities form on the biofilm. Seeding dispersal is observed in the biofilms of many bacteria such as *P. aeruginosa*, *Aggregatibacter actinomycetemcomitans, Serratia marcescens* and *Staphylococcus aureus* [14, 69, 72]. Passive detachment can take the form of sloughing and erosion. Sloughing refers to the abrupt detachment of a large portion of the biofilm, while erosion refers to the detachment of a small amount of bacteria from the biofilm [69, 72]. Planktonic bacteria released into the environment during both active and passive detachment promote the formation of new biofilms in other regions [16].

Bacterial Surface Elements Involved in Biofilm Formation

The initial contact of *E. coli* cells with the surface occurs, as in other bacterial species, through physicochemical and electrostatic interactions [73]. When the equilibrium between the bacteria and the repulsive and attractive forces of the surface begins to be disturbed, the *E. coli* cells attach reversibly to the surface. This attachment can sometimes be disrupted by bacterial movement and sometimes by fluid flow; in such cases, the bacteria are reabsorbed into the planktonic phase [40]. Flagella allow the bacteria to overcome the repulsive force and spread on the substrate surface, aiding the initial contact of the cells with the surface [74, 75]. Although flagellar motility is an important factor for adhesion, it is not a mandatory requirement. Bacteria without flagella can also adhere to surfaces by expressing intact adhesion factors [40].

Irreversible attachment to the surface leads to immobilization of *E.coli* cells, and flagella synthesis is suppressed during this process. Several small molecules, including cyclic diguanylic acid (c-di-GMP), are responsible for the transition of planktonic bacteria to biofilm form. The amount of c-di-GMP, which has a low concentration in the planktonic form, increases during biofilm formation [17]. Surface adhesins, known as type- 1 fimbriae/pili and curli, also play a role in ensuring irreversible adhesion [73].

Type 1 fimbriae or pili of *E.coli* cells are required for binding to various abiotic surfaces and receptor molecules on the surfaces of eukaryotic cells [17, 40]. Type 1 fimbriae is encoded by the *fim* operon, which consists of the *fimAICDFGH* genes. The major fimbrial subunit FimA is encoded by the *fimA* gene. FimH, an adhesin that mediates the binding of type 1 fimbriae to mannose-containing receptors, is encoded by the *fimH* gene [76]. With the onset and initial development of biofilm formation, the expression of *fim* genes is induced [17]. Type 1 fimbriae alter the physicochemical properties of the bacterial surface and affect surface adhesion by mediating the reduction in the amount of many proteins, including OmpA, OmpX, Slp and TolC, in the outer membrane [77]. Surface contacts mediated by type 1 fimbriae are a physiological adaptive signal that reduces

the amount of OmpX [78]. In the absence of OmpX, type 1 fimbriae and exopolysaccharide production increase and bacterial motility decreases [40, 77]. The roles of *E. coli* porin proteins in biofilm formation were also investigated [79]. In the study, *ΔompA*⁻, *ΔompC*⁻ and *ΔlamB*⁻ mutant strains of wild type *E. coli* W3110, which did not show biofilm formation at acidic, neutral, and alkaline pH values, formed biofilms of varying thickness at the pH values studied. In the same study, the *ΔompT*⁻, *ΔompG*⁻, *ΔompF*⁻ and *ΔphoE*⁻ mutant strains were also examined, but no biofilm formation was observed in these mutants [79].

Another surface adhesion formed by many Enterobacteriaceae is the curli fimbria [40]. Curli fimbriae are encoded by the *csgBAC-csgDEFG* operons, which are controlled by CsgD, the master regulator of many biofilm-related genes (Figure 2) [80]. The structural components of Curli (CsgB and CsgA) are encoded by the *csgBAC* operon, while the Curli export mechanism (CsgE-G) and CsgD are encoded by the *csgDEFG* operon [40, 81]. In addition to the EnvZ/OmpR two-component system, several transcriptional regulators are involved in the regulation of curli expression. These transcriptional regulators (RpoS, CpxR, H- NS, RcsCDB, IHF, MlrA, Crl) respond to many different environmental stress factors, including osmolarity, temperature, oxygen levels, and pH. Curli fimbriae increase initial cell-surface interactions and cell-cell interactions, supporting biofilm formed on abiotic surfaces [40].

In addition to type 1 fimbriae and curli fimbriae, conjugative pili (F-pilus) also play a role in enhancing interaction with the bacterial cell surface [40]. The F-pilus, which is present in Gram-negative bacteria and enables the transfer of genetic material through the formation of a conjugation bridge between bacterial cells, also provides random attachment to abiotic surfaces, stabilizing the biofilm structure and thus supporting the initial adhesion and maturation of the biofilm [40, 82].

Bacterial cells irreversibly attached to the surface begin to coalesce through cell-cell interactions. When the cells come together, production of the extracellular matrix begins, which forms the 3D structure of the biofilm and ensures its maturation [17]. The maturation phase of the biofilm is achieved through the involvement of autotransporters and EPS (bacterial extracellular polysaccharides) [17].

Autotransporters are proteins that can be localized to the outer membrane independent of the presence of auxiliary proteins [40]. Antigen 43 (Ag43), encoded by the *flu* gene is a protein found in the outer membrane of *E. coli* cells. Ag43 is a self-recognizing autotransporter protein that contributes to the adhesion of cells to each other. Ag43 contributes to the 3D development of biofilm by enabling autoaggregation and cell aggregation in liquid cultures [54]. At the same time, Ag43 promotes mixed biofilm formation of bacteria such as *P. aeruginosa* and *E. coli*. Because it is expressed in different types of bacteria. The increase in the amount of

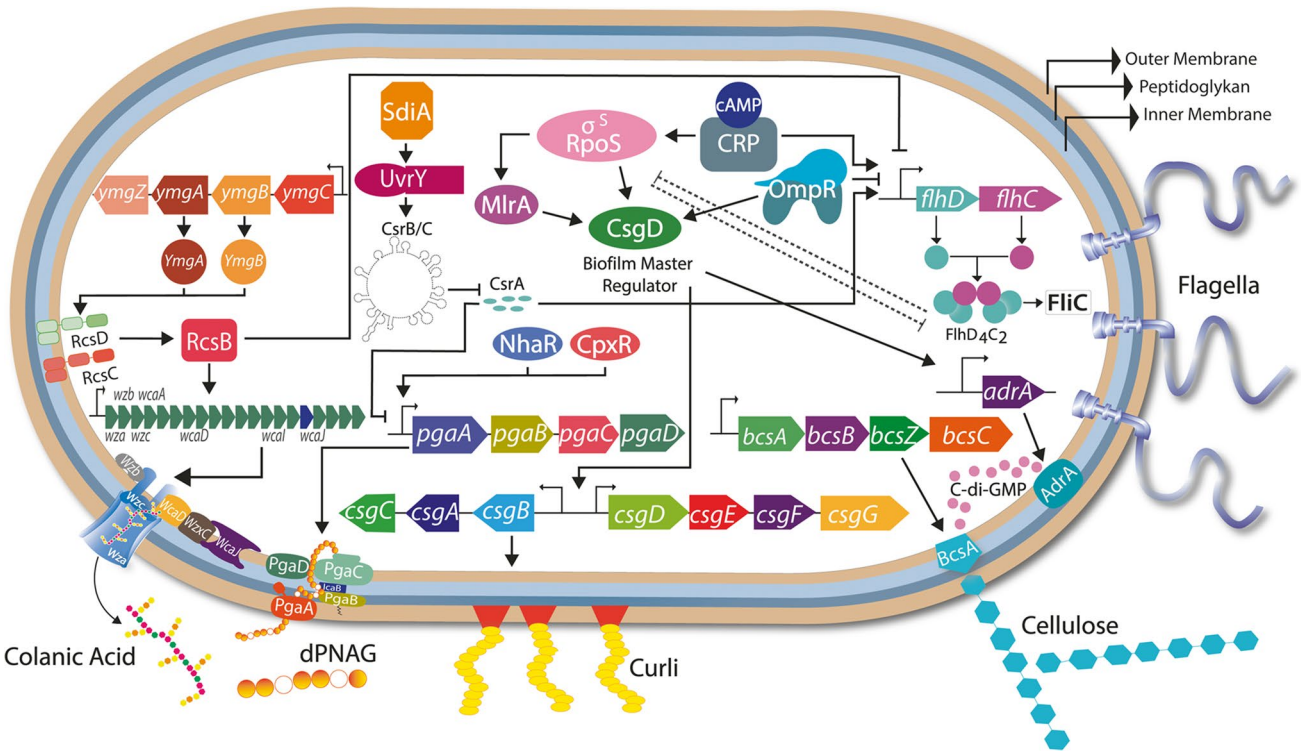

Figure 2 Key genes and their products involved in the regulation of biofilm formation in *E. coli*

Ag43 plays an important role in the formation and maturation of biofilms on abiotic surfaces [40].

AidA and TibA are other autotransporter proteins involved in biofilm formation. Expression of these proteins, usually found in virulent *E. coli* strains, supports biofilm formation on abiotic surfaces and ensures autoaggregation [28]. Ag43, AidA and TibA are self-assembling proteins that cause cell aggregation and promote biofilm formation. These proteins can establish heterologous interactions among themselves and promote the formation of mixed bacterial clusters [28, 40].

With the expression of autotransporters and the expression of matrix polysaccharides, the formation of the biofilm matrix necessary for biofilm maturation occurs [40]. The biofilm matrix provides an environment that protects the bacteria within it from external influences and adverse conditions (such as desiccation, biocides, antimicrobials, metal cations, ultraviolet radiation, protozoa, and host immune defenses) [83].

Bacteria in the biofilm matrix secrete polysaccharides that form and also serve as structural support for the biofilm. There are three major exopolysaccharides in *E. coli* biofilms; ß-1,6-*N*-acetyl-D-glucosamine polymer (PGA), cellulose, and colanic acid. In addition to these polysaccharides, lipopolysaccharide and capsules of cell surface polysaccharides are also important factors in the formation of *E. coli* biofilms [17, 83]. PGA not only plays a role in cell-cell adhesion, binding cells to surfaces, but also functions as an adhesin that stabilizes the *E. coli* biofilm. The *pgaABCD* (or *ycdSRQP*) operon in *E. coli* encodes proteins related to the synthesis, transport, and localization of the PGA polymer [17, 40]. PgaA and PgaB are required for PGA transport, while PgaC glycosyltransferase and PgaD are required for PGA synthesis (Figure 2). In general, as with many bacterial exopolysaccharides, PGA synthesis is allosterically activated by c-di-GMP [84].

Cellulose, another exopolysaccharide, ensures that biofilm formation at the air-liquid interface is hard and firm [17, 40]. Microbial cellulose, produced by connecting β-(1,4) bonds, is a linear polymer of glucose [85]. Cellulose is encoded by the *bcsABZC* and *bcsEFG* operons. In *E. coli*, the cellulose synthase complex consists of nine subunits, with seven of them located at the inner membrane [86]. BcsA and BcsB are part of the cellulose synthase complex. BcsA is a glycosyltransferase embedded in the membrane, whereas BcsB is a periplasmic protein associated with the inner membrane [85–87]. BcsA, the basic catalytically active cellulose synthase subunit, polymerizes uridine diphosphate (UDP)-activated glucose (UDP-Glc) to cellulose via an intracellular glycosyltransferase domain and releases this polymer through a transmembrane channel [85]. The BcsA cellulose synthase protein is allosterically controlled by c-di-GMP binding (Figure 2) [88].

The last of the three major exopolysaccharides found in *E. coli* biofilms is the colanic acid. Colanic acid is a negatively charged capsule composed of D-galactose, D-glucose, D-glucuronic acid, and L-fucose polymers. It protects the cell from environmental influences by wrapping around the bacterial cells. Unlike most types of capsules, a significant portion of the colanic acid produced is released into the extracellular environment [40, 89]. Biosynthesis of colanic acid occurs through enzymes encoded by a cluster of 20 genes (*wza*, *wzb*, *wzc*, *wcaA*, *wcaB*, *wcaC*, *wcaD*, *wcaE*, *wcaF*, *gmd*, *wcaG*, *wcaH*, *wcaI*, *cpsB*, *cpsG*, *wcaJ*, *wzxC*, *wcaK*, *wcaL* and *wcaM*) called *wca* [90, 91]. This gene cluster is induced by the three-component system RcsCDB in the presence of the transcriptional regulator RcsA (Figure 2). Colanic acid can impair initial bacterial attachment, but in advanced stages of biofilm formation it is upregulated and its production contributes to the development of a mature biofilm structure [40, 92]. In addition, some studies have shown that colanic acid capsule expression can inhibit biofilm formation of *E. coli* strains by masking autotransporter adhesins such as Ag43 and AidA [28].

Another component contributing to the biofilm phenotype are the capsular polysaccharide (K antigen) and the lipopolysaccharide (LPS, or O antigen). Capsular polysaccharide K antigen and lipopolysaccharide O antigen are surface polysaccharides specific for *E. coli* strains. Lipopolysaccharide is found on the outer membrane of Gram-negative bacteria and is a glycolipidic polymer also known as endotoxin [17, 40]. Over 50 genes, some of which are grouped into large operons, are involved in the synthesis and assembly of LPS on the cell surface. In studies with LPS-deficient *E. coli*, it was found that adhesion to abiotic surfaces and biofilm capacity decreased [40]. Another study reported that decreased LPS expression by mutation of the *rfaH* gene abolished the masking effect of LPS on *E. coli* adhesins, allowing initial adhesion and/or biofilm formation [93]. These studies show that LPS can enhance or reduce biofilm formation through interaction with cell surface adhesion factors [40]. Capsular polysaccharides, on the other hand, are high molecular weight structures that hold the cell and surround the surface. The *E. coli* capsule indirectly contributes to biofilm formation by protecting surface adhesives [40].

Environmental Signals and Regulatory Networks Involved in the Formation of *E. coli* Biofilms

Signals

a) **Mechanical Signals**

A bacterial cell with a smaller distance to the surface can switch between a static and a planktonic lifestyle. This suggests that surface sensing is necessary for biofilm formation [56]. Flagella not only promote surface adhesion by helping to overcome repulsive forces near the surface and increas-

ing surface proximity, but they are also involved in surface perception [94]. In *E. coli*, the flagellar master operon *flhDC* is responsible for controlling flagella synthesis. A heteromultimeric complex ($FlhD_4C_2$), which functions as a transcriptional activator and is formed by FlhDC proteins, regulates the expression of genes involved in flagella synthesis [56, 95]. Data from transcriptional profiling studies show that biofilm matrix synthesis and flagellar gene expression are regulated in reverse order. This regulation may occur by recognising the contact of flagella with the surface. When the flagella reach the surface, the rotation of the stators in the flagella is inhibited, and the flagellar motor stops rotating [56]. The sudden change in ion flux due to the arrest of the flagella can lead to hyperpolarization within the cell. While respiration and ion output are constant, sudden changes in ion flux due to the arrest of the flagella can cause hyperpolarization within the cell. Electrochemical stimuli that are mechanically triggered are thought to trigger the intracellular signals required for biofilm formation in a manner that has not yet been clearly demonstrated [94, 95]. In the absence of a flagellar motor, interaction with the surface is not transmitted [56].

b) **Nutrient Availability and Metabolic cues**

The type and amount of nutrients available regulate the response of bacteria to their environment. The energy cost of forming a biofilm and then spreading from it requires that the bacteria analyze the nutrient status of the medium. There are some nutrient signals that influence biofilm formation [56, 96].

Glucose and its catabolic repression are one of these signals. Glucose is a carbohydrate that plays an important role in the metabolism of many organisms living on Earth. In some bacteria, glucose and similar carbohydrates trigger biofilm formation, while in others they inhibit surface adhesion. Environmental suppression of glucose and catabolite repression inhibits biofilm formation in several *E. coli* strains. In *E. coli*, the suppressive effect of glucose takes the form of catabolite suppression via the cAMP-CRP system [56, 96]. The *bssS* and *bssR* present in *E. coli* act as regulators of some genes involved in catabolite repression. BssS and BssR can significantly suppress biofilm formation by several systems (such as RpoS, CsrA, CreC, and CRP) that are modulated by glucose. Expression of CsrA leads to suppression of glycogen metabolism and activation of the *flhDC* operon. At the same time, the expression of CsrA decreases dramatically with the onset of biofilm formation. If CsrA is active after biofilm maturation, expression of flagellar genes may occur, leading to detachment of cells from the biofilm. CsrA regulates PGA production post-transcriptionally by acting indirectly on the *pgaABCD* operon (Figure 2). Pyruvate and indole, used as nitrogen and carbon sources in nutrient-poor environments, are obtained by the degradation of tryptophan by tryptophanase. Indole stimulates biofilm formation in *E. coli* and many Gram-negative bacteria [56, 97]. Indole is a universal biofilm signal and helps cells detect and respond to nutrient deficiencies [56].

Polyamines such as putrescine, spermidine, and norspermidine are organic molecules that contain at least two amine groups and are positively charged at neutral pH. Polyamines are essential for cell development and can act as signals that modulate biofilm formation [56]. *E. coli* possesses one spermidine uptake transporter, PotABCD, and five putrescine transporters (PotE, PotFGHI, PlaP (YeeF), PuuP and YdcSTUV). PotD of the PotABCD transporter is required for biofilm formation [98]. In studies with *E. coli* cultures, exogenous putrescine was found to stimulate cell viability and biofilm formation, and spermidine supported biofilm formation [98, 99].

c) **Inorganic Molecules**

Inorganic molecules such as iron and phosphate have effects on biofilm formation. CsgD represses the transcription of *fecR*, which is responsible for the transcriptional activator of iron uptake genes in *E. coli*. This indicates that biofilm formation is inversely regulated by iron uptake [56]. Phosphate in the medium increases cell viability in *E. coli* biofilms [100].

d) **Osmolarity**

Osmolarity acts as a regulator of biofilm formation in many bacterial species. Depending on the type of osmolyte present in the environment, biofilm formation is affected. For example, culturing *E. coli* cells in a medium containing NaCl causes the transcription factor CpxR to repress the transcription of curli genes, whereas the same effect does not occur in a medium containing sucrose. This indicates that ionic strength rather than osmolarity is used as an environmental signal. Addition of NaCl to the medium activates transcription of the *pga* operon, which contains the genes for the proteins that enable PNAG synthesis. Different salt concentrations in the environment have different effects on *E. coli* biofilms. This effect of osmolarity, which has a differential impact on bacterial biofilm formation, is explained by the different physiology of bacteria [56].

Secondary Messengers and Small Molecules

Small molecules such as cyclic di-GMP, acetyl phosphate, Alarmone ppGpp, *N*-acetyl-glucosamine and *N*-acetylglucosamine-6-P play a role in the transition from the planktonic form to the biofilm form [40].

a) **Cyclic di-GMP**

The synthesis of cyclic di-GMP (c-di-GMP), a secondary messenger, is carried out by diguanylate cyclases, while its degradation is ensured by phosphodiesterase [40, 101].

Activation of CsgD in *E. coli* leads to stimulation of *adrA* (diguanylate cyclase) and cellulose synthesis is regulated. This regulation occurs when diguanylate cyclase activates BcsA and produces c-di-GMP (Figure 2). Once BcsA is activated, it ensures the formation of bacterial cellulose [102]. C-di-GMP affects many cellular functions in the cell. One of them is the regulation of movement via YcgR and the other is the regulation of biofilm formation by stimulating some bacterial structures such as the curli fimbriae. High concentration of c-di-GMP is detected in *E. coli* during biofilm formation, while low concentrations ise detected during motility [103, 104].

b) **Acetyl Phosphate**

Accumulation of acetyl phosphate (AcP) in the cell occurs when there is an abundant carbon source in the environment. Local oxygen starvation, which occurs when bacteria come into contact with a surface, is a signal that causes increased intracellular AcP levels. The increase in AcP level leads to high expression of colanic acid and type 1 pili and low expression of flagellar genes, which promotes biofilm maturation. This state of expression promotes biofilm maturation [40, 105].

c) **Alarmone ppGpp**

Guanosine 5'-diphosphate 3'-diphosphate (ppGpp) and guanosine 5'-triphosphate 3'-diphosphate (pppGpp) are small nucleotides called alarmones. Alarmones are involved in environmental adaptation and regulate gene expression through their action on response regulators [106]. Amino acid levels in the *E. coli* environment are constantly changing, and amino acid levels that are reduced enough to affect growth trigger the binding of ppGpp to RNA polymerase. This downregulates stable RNAs, which results in reduced protein synthesis [107]. Accumulation of the alarmone amount affects the transcription of about 500 genes [108, 109]. ppGpp, which is responsible for performing the stringent response of *E. coli*, affects one of the *fimB* promoters during the stringent response, resulting in decreased *fimB* transcription and thus a decrease in the amount of type 1 fimbriae [106]. It has been shown that ppGpp can indirectly increase the expression of *dsrA* (small regulatory RNA) and *iraP* (anti-adapter protein) by reducing rRNA transcription and increasing RNA polymerase availability [110]. In turn, DsrA indirectly causes suppression of *fimB* expression by impairing RpoS [107]. However, in planktonic bacteria in nutrient-poor environments, ppGpp signaling is thought to induce the production of type 1 fimbriae and promote colonization of surfaces with better conditions [40].

d) ***N*-Acetyl-Glucosamine and *N*-Acetylglucosamine-6-P**

PGA, a subunit of *N*-acetylglucosamine, is an extracellular polysaccharide that promotes cell-cell adhesion, stabilization of biofilm formation, and attachment to solid surfaces in *E. coli*. *N*-acetylglucosamine-6-P is a signal that can induce the cell to produce sticky molecules. *N*-acetylglucosamine-6-P is used by *E. coli* as a precursor in the biosynthesis of peptidoglycan and lipopolysaccharide or as a carbon source [40]. This extracellular polysaccharide structure also protects cells from the effects of environmental factors by reducing the effect of antimicrobial agents on cells [111]. Addition of the mucin sugar *N*-acetyl-glucosamine to the medium or mutation of the regulatory protein NagC was shown to reduce biofilm formation in AIEC strain LF82 [112].

Regulatory Networks

a) **Two Component Regulatory *cpxRA* system**

The cytoplasmic regulator CpxR and the sensor membrane protein CpxA form a two-component regulatory system, CpxRA [113]. This system regulates gene expression under stress conditions such as high pH, overproduction and misfolding of membrane proteins and is involved in the adaptation of bacteria to stressful environmental situations [114, 115]. Initial attachment of *E. coli* to abiotic surfaces induces membrane disruption, which activates the *cpx* system in response to the surface sensor CpxR [116]. This process is referred to as surface recognition. Activation of CpxR leads to suppression of motility and chemotaxis genes, accelerating the transition of cells to the stable structure of the biofilm [40]. Contact of cells in developing *E. coli* biofilms leads to enhanced induction of the cpx system. In this way, the cpx system makes an important contribution to biofilm maturation [40].

b) **Two Component Regulatory *rcs* System**

The two-component regulatory system consists of Rcs, the cytoplasmic reaction regulator RcsB, and the membrane-associated proteins RcsC and RcsD [117]. The sensor kinase RcsC detects desiccation, changes in osmolarity, and membrane disruption (when growing on a solid surface) and is therefore essential for nor-

mal biofilm development in *E. coli* (Figure 2) [40, 118]. Flagellar gene expression is masked in *E. coli* by the bicomponent *rcs* [117]. In studies using mutant *rcsC* and *rcsB* cells, it was found that the initial attachment of these mutant cells was not affected, suggesting that this regulon is involved in biofilm maturation rather than the early stages of biofilm [40].

c) **Two-Component Regulatory EnvZ / OmpR System**

The EnvZ/OmpR two-component system regulates the porin proteins *ompC* and *ompF* at the transcriptional level in response to changes in external osmolarity [119]. EnvZ/OmpR activity represses flagellar gene expression upon a moderate increase in external osmolarity and can promote adhesion by activating curli after primary adhesion [40]. The regulator CsgD activated by phosphorylated OmpR induces curli expression, and curli expression is indirectly regulated by OmpR [40, 120].

d) **RpoS and H-NS**

RpoS and H- NS are involved in the regulation of biofilm formation in response to environmental conditions [40]. H-NS is involved in the regulation of several genes in the genome as well as gene regulation associated with many biofilms, such as type 1 fimbriae and flagella. H- NS acts on the sigma factor RpoS and regulates the expression of RpoS-dependent genes [40, 121]. This regulation can take several forms. H-NS can bind to the promoter to which RpoS binds, preventing its binding, which indirectly represses translation of RpoS or stimulates turnover of RpoS [40].

Quorum Sensing

Quorum sensing (QS) is a chemical signaling system that modulates gene expression in response to the presence of small signaling molecules called autoinducers or quormones secreted by cells. The intensity of autoinducers specific to different species is directly proportional to the cell population present in the medium. The amount of autoinducers increases with population density and regulates biofilm formation and gene expression during maturation via cell-cell signaling [17, 122]. At high concentration, AI interacts with a protein that increases motility, expression of thermostable toxins and fimbriae, and modulates gene expression of virulence factors [123]. There are three types of autoinducers in Gram-negative bacteria, AI-1 (*N*-acyl homoserine lactone (AHL)), AI-2 (furanosyl borate diester) and AI-3 [124, 125]. One of the two elements that make up the AI-1 regulatory system is LuxI (AI-1 synthase) and the other is LuxR (AI-1 response regulator). LuxI and LuxR are involved in controlling some processes such as biofilm formation and virulence [17]. *E. coli* cannot synthesize AHL, but thanks to its genome, it encodes the *luxR* homolog, *sdiA* (the AI-1 sensor). AHL caused by other bacteria is recognized by *luxR* encoded by *sdiA*. SdiA also increases motility, virulence, and biofilm formation of *E. coli* by positively regulating the *csrA* and *uvrY* genes [40]. Al-2 is responsible for bacterial communication QS within and between species. It significantly increases biofilm biomass through the QS regulator (YgiU) of AI-2. YgiU regulates flagellar movement through the QseBC two-component system. The QS regulator YgiU activates QseB via *flhDC* (stimulates MotA and FliA), thereby increasing biofilm formation and controlling motility. In addition, YgiU controls the induction of curli with *crl* and the stimulation of motility with *csrA* [17]. AI-3, another autoinducer involved in QS, is produced by the microbial gastrointestinal flora and detected by histidine sensor kinases of enterohemorrhagic *E. coli*. The component of QseBC, one of the two-component systems characterized in *E. coli*, activates the primary regulators of the flagella regulon through QseB after QseC detects AI-3 in the environment. Thus, it causes swimming motility so EHEC can move toward the intestinal epithelial layer [126, 127].

Detection of Biofilm Formation

There are many methods to evaluate biofilm production. The standard test to detect biofilms is the crystal violet assay (CV), which quantifies the dye bound to cells on polystyrene and other hydrophobic substrates. However, the CV assay has some limitations, there are several washing steps that can lead to cell loss, and most importantly, the biofilm must be broken up to obtain the desired result [128]. The microtiter plate method is one of the most commonly used standard methods and is preferred because it is more reliable than the tube method and the Congo red agar method [129].

a) **Tube Method**

The tube method (TM), a qualitative biofilm detection method, was defined by Christensen et al. in 1982 [130]. In this method, bacterial isolates are inoculated into tubes with a medium, usually LB or TSB. The tubes are then incubated for 24 hours. After incubation, the medium in the tube is poured off and washed with phosphate buffered saline (PBS). This washing process removes planktonic bacteria that are not involved in the formation of the biofilm from the environment. After the washed tubes are dried in the inverted position, the same volume of dye (e.g., crystal violet, safranin) as the spilled medium is added to the tube and a period of time is allowed for staining of the biofilm. After staining, the tubes are washed again with PBS and dried.

After drying, the dye that stained the biofilm structures is dissolved using a solvent (e.g. methanol, acetic acid, ethanol) [131]. Subsequently, the biofilm production of all strains is evaluated by measuring the absorbance of the dissolved dye [132].

b) **Congo Red Agar Method**

The Congo Red Agar (CRA) method, defined by Freeman et al. (1989), is a simple qualitative method in which biofilm-producing microorganisms are determined by the color changes of colonies inoculated into the medium. CRA medium is obtained by mixing 0.8 g/L Congo red and 36 g/L sucrose into 37 g/L Brain heart infusion (BHI) agar. After incubation at 37°C for 24 to 48 hours, the colors formed by the colonies are assessed. Curli production by *Salmonella* and *E. coli* strains is measured qualitatively by staining colonies grown in CRA medium. Congo red can bind to other cellular properties, including cellulose, so this should also be considered in the evaluation. A four-color reference scale is used when evaluating the color of colonies. According to this scale, black and blackish-brown colonies are defined as biofilm-positive strains, while burgundy and red colonies are characterized as non-biofilm-generating strains [133–135].

c) **Microtiter Plates (MTP)**

Microtiter plates (Figure 3) are the most common method for assessing biofilm formation. This method was originally developed by Madilyn Fletcher for the study of bacterial attachment [136, 137] The method generally allows the detection of biofilms formed on the surface of the wells of polystyrene MTP. For this purpose, a certain concentration of bacterial culture is added to the wells and the microplates are incubated for a certain period of time without shaking. This period can vary from 1 to 4 days depending on the ability of the bacteria to form biofilms. After incubation, the medium in the wells is removed and several washes are performed to clean the biofilm mass formed in the wells from non-adherent cells (planktonic). Then, the adherent cells are stained with various dyes (such as crystal vio-

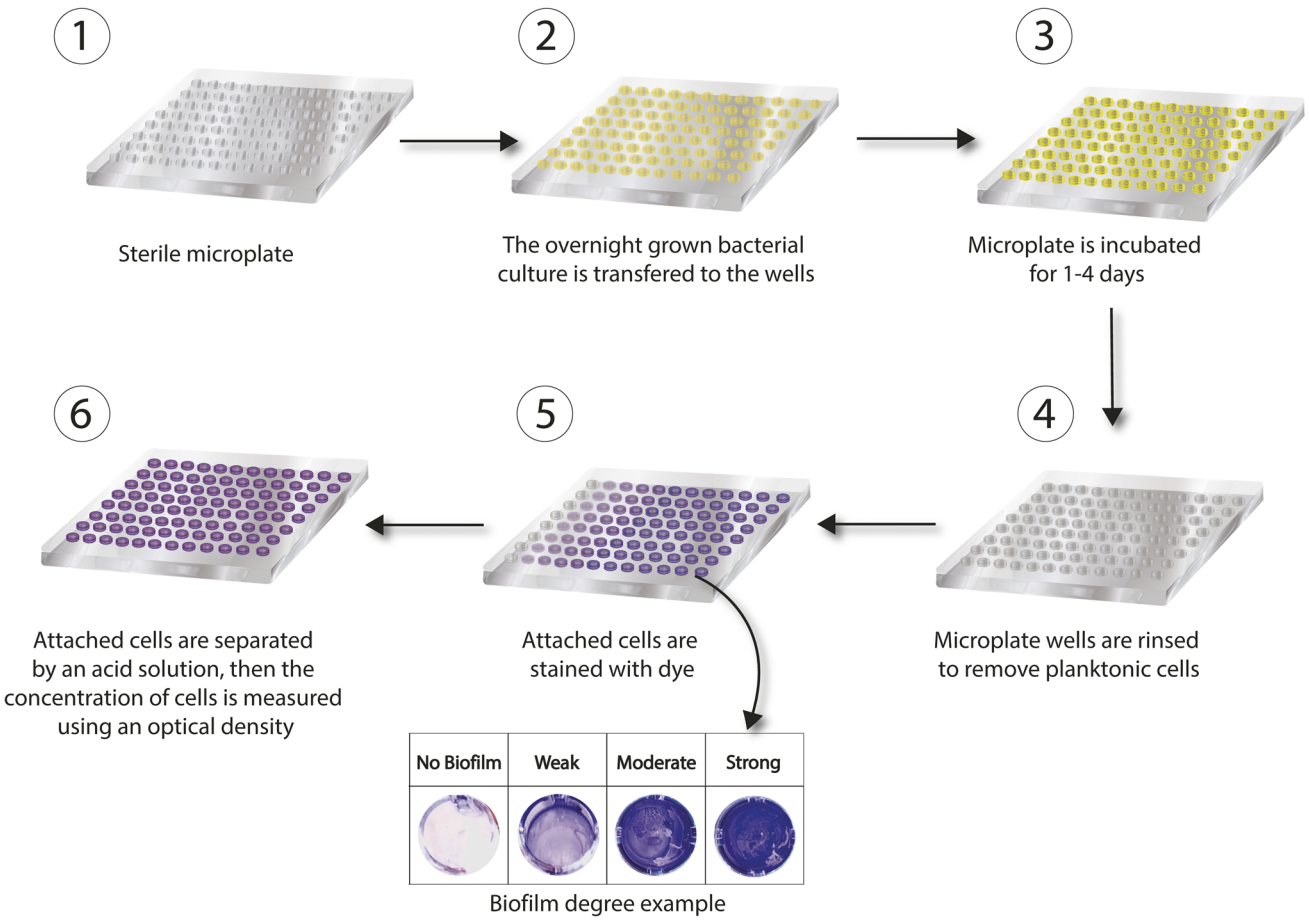

Figure 3 Microtiter plates method

let, safranin) and separated with solvents such as acetone and methanol. The biofilm mass formed by the cells is then measured at specific wavelengths (in the range of 500-600 nm), and the degree of biofilm formation (strong, moderate, weak, absent) is determined based on the measurement results [132].

In the MTP assay, biofilm biomass and total associated biomass are measured and evaluated. This means that cells that have previously settled at the bottom of the wells and are taken up by the extracellular polymeric substances (EPS) into the biofilm biomass may also be included in this biofilm biomass. Therefore, biomass independent of biofilm formation can be added to this biomass. This makes it impossible to measure biofilm biomass accurately [136].

There are some studies comparing the methods (TM, CRA and MTP) to detect biofilm formation [129, 131, 138]. In these studies, clinical isolates [129] and uropathogens [131, 138] were used. The results of the studies show that MTP is the best method for detecting biofilm formation [129, 131, 138].

d) **Calgary Biofilm Device (CBD)**

The Calgary Biofilm Device, which is preferable to the microplate method, was developed to be used in biofilm mass assessment and antibiofilm studies [139]. The easy-to-use device also reduces the risk of contamination and allows results to be obtained in a short time [139].

The device was designed with a very simple approach and has pins that are compatible with 96-well microtiter plates. After closing the lid with these pins over the wells containing the medium and the bacterial inoculum, it is allowed to incubate for a certain time. As a result of the incubation, the formation of a biofilm on the pins is observed. The observed biofilm formation is due to adherent cells, not collapsed cells. In this way, the biofilm structure formed by the adherent cells can be easily separated from the surrounding environment. Biofilms on CBD immersed in fresh medium can be tested against various antibiotics, chemicals and different biofilm agents [139–141].

e) **Drip Flow Biofilm Reactor**

The drip flow biofilm reactor designed by Darla Goeres et al. (2009) is a device with four parallel chambers with ventable lids [142]. Each chamber of the device has a coupon (like a microscope slide) on which bacteria can form a biofilm. The cell suspension or medium, supplied via the injector from the lid area at the top of the device, is directed into each compartment of the device. When the device is turned on, the reactor tilts 10° from horizontal, with the fluid running along the coupons [136, 143]. In this way, a biofilm is formed on the surface of the coupons, which is subsequently evaluated. The drip flow reactor is shown in Figure 4.

This reactor has many advantages. Some of these advantages are: It requires less space, is easy to operate, and can be used simultaneously with different surface

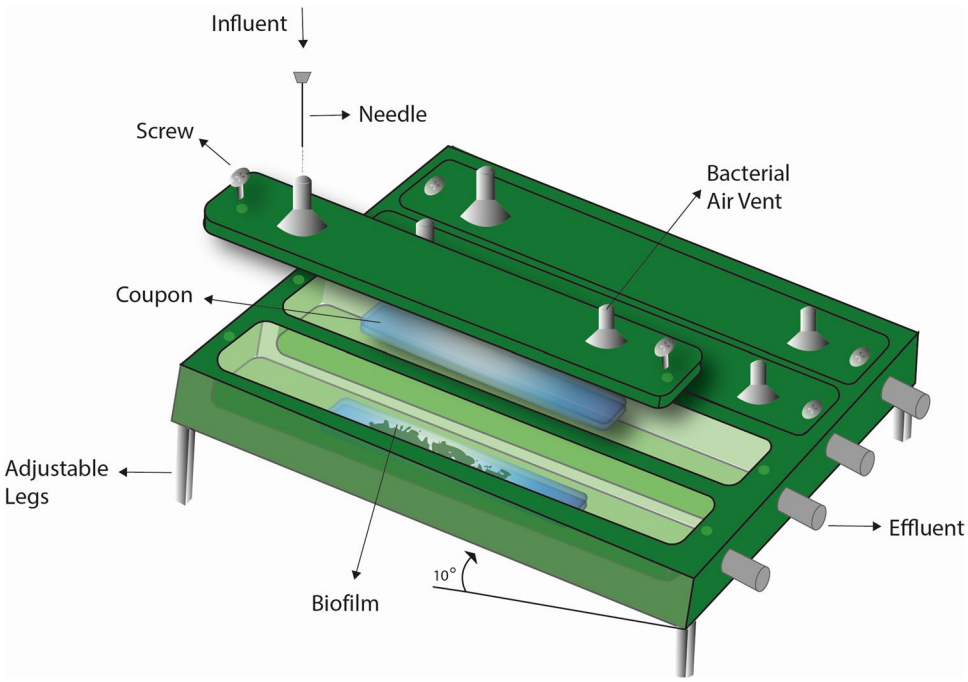

Figure 4 Drip flow reactor

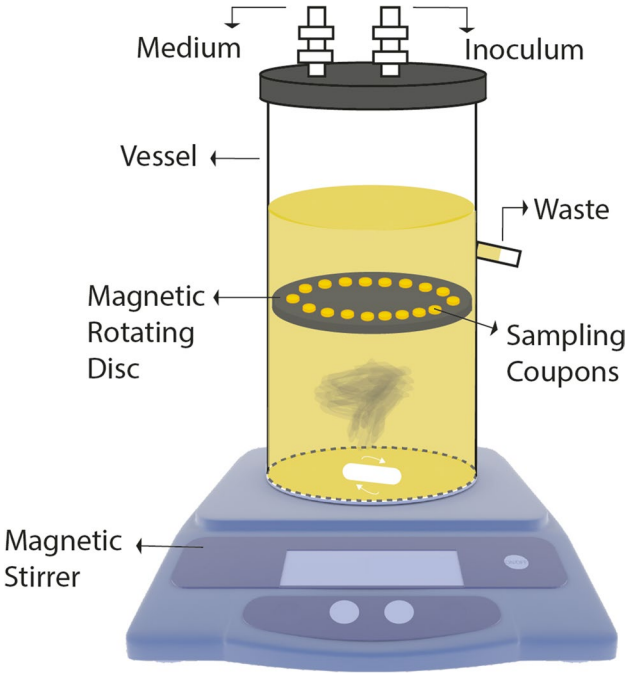

Figure 5 Rotating disc reactor

materials. Therefore, this reactor is often used for various experiments [136].

f) **Rotary Biofilm Reactors**

Rotary biofilm reactors can be divided into three types. These are the circular reactor, the disc reactor, and the concentric cylinder reactor [136]. An example of a disc reactor is shown in Figure 5.

The rotating circular reactor was developed in 1968 and consists of a rotating inner cylinder and a stationary outer cylinder [144]. The rotation frequency of the inner cylinder is controlled by a motor so that constant cut surfaces can be achieved [145]. The coupons, which are used as surfaces for evaluating biofilm development, can be taken from the system so that the biofilm can be observed chemically, biochemically, and microscopically [136]. By using these rotating reactors, it is possible to evaluate the efficacy and action processes of the antimicrobial agents used, as well as to gain knowledge about biofilm formation in drinking water systems [136, 146].

The spinning disc reactor has a special design that can accommodate multiple coupons. Due to the magnetic attachment, it can achieve its rotation speed with a magnetic stirrer [136, 147].

The concentric cylindrical reactor with four concentric chambers has four cylindrical sections that can be rotated in these chambers at different speeds. Thanks to this configuration, different shear stresses can be tested simultaneously depending on the radius of the surface [136, 148]. The reactor allows testing different types of bacteria or the same bacterial suspensions thanks to its chambers where independent samples can be taken and fed. One of the advantages is also that different hydrodynamic conditions can be tested in each chamber [136, 149].

g) **Flow Chamber Model**

The flow chamber model is a system through which a fresh medium is constantly flowing. The system traps the air bubbles formed in the growth medium and in the flow chamber and removes them from the environment. A microscope cover glass is attached to the top of the chamber using a silicone adhesive or similar [136].

The geometric shape of the chambers ensures that the flow of the growth medium is turbulent or laminar. This affects the distribution of nutrients and removal of waste materials [136, 150]. Bubble traps, on the other hand, ensure the removal of stray bubbles that interfere with biofilm growth [136]. Flow-through chambers are preferred as often as microtiter plates because they provide a controllable and repeatable system similar to those in the natural environment. In addition, flow chambers, which offer lower yields than microtiter plates, do not require a washing step to remove unbound cells [136].

h) **Microfluidic Devices**

Closed systems in which microbial biofilms interact with hydrodynamic environments are called microfluidic devices. The channels in the devices have been fabricated to study the effects of many factors on biofilm formation [136, 151]. These devices can be made from many different materials [152]. These devices were designed for specific purposes, such as mimicking air-liquid interfaces. Microfluidic devices are not consistently used in biofilm studies. This is because the general applicability of microfluidic techniques is difficult, the methodology is difficult, and much experience is required for successful use [136].

Imaging Biofilms - Microscopic Methods

Biomass, dynamics, complexity, and cell viability of the biofilm can be determined using many imaging techniques [128, 136]. The presence of the biofilm can be verified by visualizing the 3D structure of the biofilm using methods such as fluorescence microscopic examination, atomic force microscope, light microscope, scanning electron microscope (SEM), and confocal laser scanning microscope (CSLM) [128]. However, SEM is an expensive method, and biofilm

is difficult to quantify with SEM. With CSLM, high-speed calculations and fluorescence staining methods can be used to understand the distribution and heterogeneity of cells in the biofilm [128, 153, 154].

a) **Light Microscopy**

A good technique for visual identification of biofilm formation is light microscopy. The total mass of the biofilm and the cell mass of the biofilm are closely related to the light absorption of the biofilm [136]. The volume and structure of biofilms were monitored using the light microscope according to the method described by De Carvalho and Da Fonseca, eliminating the need for more expensive methods [155]. The basic idea of this method is the linear relationship between the density of a pixel in biofilm images taken in the x-y plane and the number of cells corresponding to it in the z direction. In this way, information about the thickness of the biofilm can be obtained. The reasons for choosing the light microscope, which has many advantages, are mainly its low cost, simple method, and easy sample preparation. In addition to these advantages, there are also some limitations, such as low required magnification and resolution, and difficulty in discrimination in thick samples [136].

b) **Atomic Force Microscope**

Atomic force microscopy (AFM) is used to study biological samples at micrometer and nanometer scales under conditions that do not damage the product [136]. Various mechanical signals that occur during adhesion, such as adhesion forces, cause bacteria to respond [156]. Adhesion forces (intercellular, intercellular-surface, and intermolecular) can be measured by AFM [157, 158]. In an AFM study, it was shown that the increase in hardness of *Pseudomonas* biofilms was due to the production of amyloid protein [159]. AFM, which allows the study of the three-dimensional evolution of biofilms, allows the quantitative determination of the biomass of the biofilm by the amount of EPS and thickness with roughness and height analysis (Figure 6) [156].

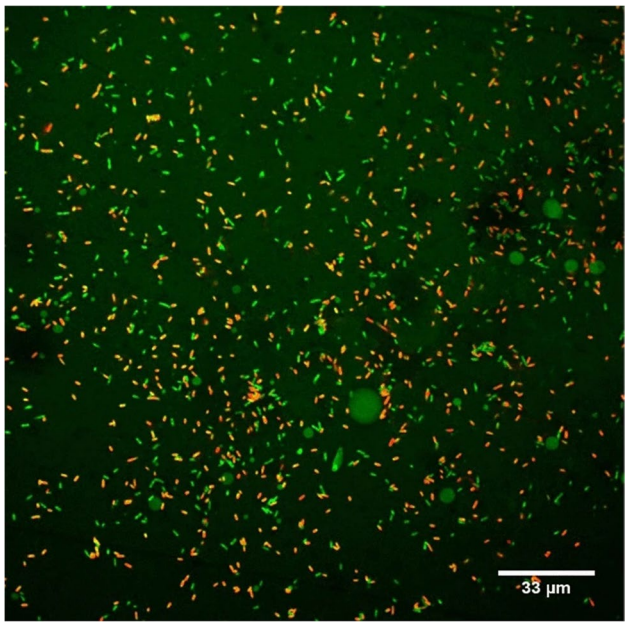

Figure 7 Confocal laser scanning microscope image of *E. coli* biofilm on glass slide [166]

c) **Confocal Laser Scanning Microscope (CLSM)**

Confocal laser scanning microscope (CLSM) is a microscope that can be used to quantitatively evaluate structural parameters such as biovolume (total volume of cells in the area of observation), thickness, and roughness. CLSM can be successfully applied to various types of biofilms in combination with a fluorescent dye (Figure 7) [156]. CLSM provides resolution at the level of single cells. As described by Thornton et al. (2011, identification of a single species (often a pathogen) in multispecies samples can be achieved using probes labeled with different fluorescent dyes (FISH followed by CLSM) [160]. The same approach can be used to

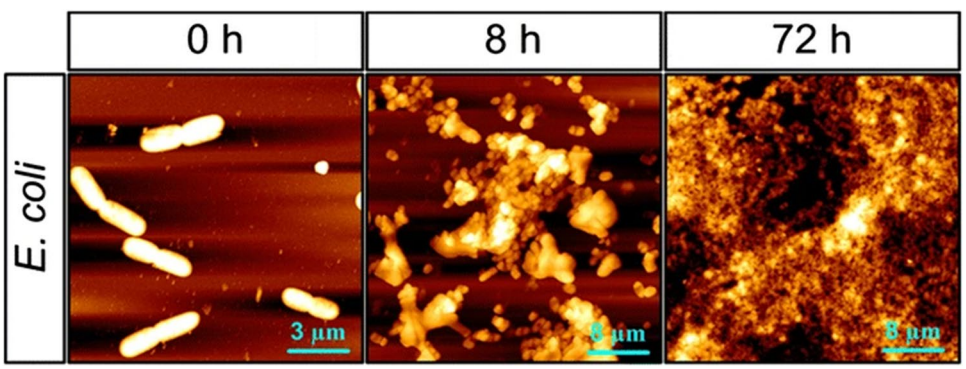

Figure 6 AFM height images showing the morphology of *E. coli* biofilms after 0, 8 and 72 h cultivation [165]

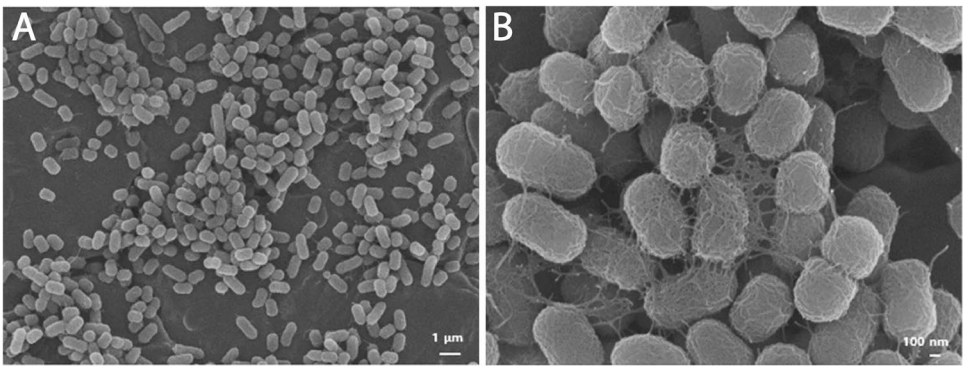

Figure 8 SEM images of *E. coli* O157:H7 biofilms on stainless steel surfaces. Magnifications and bar markers are ×10,000 and 1 µm long (**a**) and ×50,000 and 100 nm long (**b**), respectively [167]

analyze interspecies interactions in addition to assessing interspecies competition [161].

d) **Scanning Electron Microscopy (SEM)**

The absorption of electrons and their scattering on the surface form the basis of scanning electron microscopy [136]. Using SEM, the presence of EPS can be detected and the spatial structure can be studied [162, 163]. Conventional SEM and FESEM (Field Emission Scanning Electron Microscopy) are the best methods to visualize biofilms when high resolution and high magnification images are required for a good description of biofilm morphology (Figure 8) [162–164]. It can be used in biofilm studies that require evaluation of the anti-biofilm effect of the treatment/preparation, and it is important to perform a comparative analysis [136].

Future Perspectives

Biofilm formation, which contributes to the adaptation of microorganisms to environmental conditions, will continue to attract the attention of researchers in the near future due to its positive and negative effects in many different fields. With the help of new generation technologies, it will be possible to elucidate the regulatory mechanisms affecting biofilm formation, make biofilms more efficient, eliminate undesirable harmful biofilm formation, and uncover new benefits of biofilms. In this way, it will be possible to ensure that biofilms that are in harmony with nature have a better place in our lives and are used for our benefit.

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