Chapter

Antibiotic Resistance in Biofilm

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Abstract

Biofilms can be found on several living and nonliving surfaces, which are formed by a group of microorganisms, complex assembly of proteins, polysaccharides, and DNAs in an extracellular polymeric matrix. By forming a biofilm, bacteria protect themselves from host defense, disinfectants, and antibiotics. Bacteria inside biofilm are much more resistant to antimicrobial agents than planktonic forms since bacteria that are unresisting to antimicrobial agents in any way can turn resistant after forming a biofilm. Low penetration of antibiotics into the biofilm, slow reproduction, and the existence of adaptive stress response constitute the multiphased defense of the bacterium. This antibiotic resistance, which is provided by biofilm, makes the treatments, which use effective antibiotic doses on the bacterium in planktonic shape, difficult. Biofilm formation potential of bacteria appears as an important virulence factor in ensuring the colonization on the living tissues or medical devices and makes the treatment difficult. The aim of this chapter is to overview the current knowledge of antimicrobial resistance mechanisms in biofilms.

Keywords: biofilm, antibiotic resistance, bacteria, antimicrobial agents

1. Introduction

Bacteria can grow in biofilms on a wide variety of surfaces and attach to inert or alive surfaces, including tissues, industrial surfaces, and artificial devices, such as catheters, intrauterine contraceptive devices, and prosthetic medical devices, implants, cardiac valves, dental materials, and contact lenses [1, 2]. Biofilm growth confers several advantages to bacteria, including protective against hostile environments conditions such as osmotic stress, metal toxicity, and antibiotic exposure.

Biofilm-associated drug resistance and tolerance play a major role in the pathogenesis of many subacute and chronic bacterial diseases and their recalcitrance to antibiotic treatment, especially in medical device-related infections.

The definition of biofilm has been made with the development of new techniques for the direct examination of biofilms over the last four decades. Initially, a biofilm was defined as the composition of bacterial communities bound to coated surfaces in a glycocalyx matrix; subsequently, the correct definition of biofilm was made not only by considering its easily observable properties, such as cells irreversibly attached to a surface or interface embedded in an extracellular polymeric matrix material, but also by taking into account other physiological properties of these organisms such as altered growth rate and different gene expression [3].

A biofilm can be described as a microbially derived sessile community characterized by cells. These cells are irreversibly attached to a surface or interface or to each other, are inserted in a matrix of extracellular polymeric substances (EPSs) that they have produced, and exhibit an altered phenotype in terms of growth rate and gene transcription [4].

EPSs consist of proteins, cellulose, alginates, extracellular teichoic acid, poly-N-acetyl, and other organic compounds [4, 5] and play a critical role in the formation of glucosamine, lipids, nucleic acids, phospholipids, polysaccharides, and extracellular DNA (eDNA) and in physical interactions [4].

The stages that occur during the biofilm development are the initial attachment of the planktonic cell to the surface, followed by cell differentiation, EPS secretion, maturation, and dispersion of biofilm [6]. It can be summarized in three main stages: irreversible adhesion to the surface, being followed by bacterial division and production of the extracellular matrix, and, finally, disassembly of the matrix and dispersion of bacteria [2]. Quorum Sensing (QS) is one of the regulatory mechanisms that plays an important role in coordinating biofilm formation in many species but QS may not be the primary regulatory mechanism and serves as a checkpoint during the development of biofilm [6].

2. Causes of antibiotic failure in biofilm

Antibiotic resistance is the acquired ability of a microorganism to resist the effect of an antimicrobial agent and is associated with inheritable antibiotic resistance. On the other hand, antibiotic tolerance is a transient and nonheritable phenotype defined by the physiological state of biofilm cell populations. Also it can be provided by biofilm-specific characteristics that limit drug diffusion and activity [7]. For an antimicrobial agent to act on biofilm-forming microorganisms, it must overcome some factors, such as an increased number of resistant mutants, high cell density, molecular exchanges, substance delivery, efflux pump, and persistent cells.

2.1 Antibiotic penetration

Antibiotic molecules ought to penetrate throughout the biofilm matrix to impact the covered cells. The extracellular polymeric matrix influences the amount of the molecule, which is transferred to the inner layer of biofilm and interacts with an antibiotic agent, so it provides an anti-spread barrier for an antimicrobial agent. Biofilm EPS confers a physical barrier containing numerous anionic and cationic molecules such as proteins, glycoproteins, and glycolipid that can bind charged antimicrobial agents and provide shelter for microorganisms [8]. For example in *Pseudomonas aeruginosa* biofilms, Pel exopolysaccharides, an EPS component is able to spread cationic antibiotics such as aminoglycosides and, thus, provides tolerance to these molecules [9].

The adsorption sites of the matrix also limit the transportation of antimicrobial substances. Glycocalyx layer, component of EPS, can accumulate antibacterial molecule up to 25% of its weight and serve as an adherent for exoenzymes [10].

It is commonly accepted that in written materials lowered antibiotic penetration toward the EPS layer does not adequately clarify the risen resistance of microorganisms forming biofilm against most antimicrobial agents. The act of lowered antibiotic penetration in developing biofilm is not clear due to the fact that even antibiotics, which quickly disperse the biofilm, do not lead to notable cell death. It is suggested that reduction of antibiotics penetration might provide time for an adaptive phenotypic response, which can probably reduce susceptibility [11].

2.2 Accumulation of antibiotic-degrading enzymes in the matrix

The microorganisms that form biofilm are able to collect high amounts of β-lactamases in the biofilm matrix as a defense mechanism.

When *P. aeruginosa* biofilm matrix accumulates β-lactamases, it can lead to increased hydrolysis of antibiotics, such as imipenem and ceftazidime. It is demonstrated that *P. aeruginosa* PAO1-J32 biofilms have shown high promoter (ampC β-lactamases) activity, which is determined by scanning confocal laser photomicrographs [12]. Also, while ampicillin cannot reach the deeper layers of *Klebsiella pneumoniae* biofilms associated with β-lactamase activity, deletion of β-lactamase increases the amount of ampicillin that reaches the deep layer [13].

2.3 DNA in biofilm matrix

Extracellular DNA (eDNA) is a significant and common component ingredient of the bacterial biofilm matrix. The eDNA can be obtained endogenously without quorum sensing-mediated release, from the outer membrane or from the cell integrity-degraded biofilm microorganisms [14]. DNA can increase biofilm resistance to certain antimicrobial agents [15].

One of the mechanisms by which the DNA increases biofilm resistance is that it causes changes in outer membrane because DNA is an anionic molecule; it is able to chelate cations, such as magnesium ions and cause a lowering Mg^{2+} concentration in membrane. Magnesium restriction in *P. aeruginosa* and *Salmonella enterica* serovar Typhimurium is an environmental signal that induces energizing of the two-component systems PhoPQ and PmrAB to provide antimicrobial resistance [16].

These signal molecules are responsible for the rearrangement of the PA3552-3559 operon. The operon encodes to protein having enzymatic activity that attaches aminoarabinose to Lipid A part of the lipopolysaccharide layer, so it provides resistance against cationic peptide and aminoglycoside [17].

A polyamine, spermidine, localized to the outer membrane contributes to saving the cell from aminoglycosides and cationic peptides that are antimicrobial agents by lowering outer membrane penetrability for these positively charged molecules. Spermidine synthesis is another resistance mechanism induced by eDNA-associated cation restriction in *P. aeruginosa* [18].

Playing a physical role in defense against antibiotics, eDNA has also provided horizontal transfer of antibiotic resistance genes between microorganism cells forming biofilm [14].

2.4 Growth rate, stress response, and persistent cells

During growth in biofilm structures, physiological heterogeneity happens due to the occurrence of oxygen and other nutrients gradient in biofilms. This gradient is created because cells that are close to the surface of the biofilm consume obtainable nutrient sources and oxygen before the nutrients disperse into depth of the biofilm [19]. Nutrient and oxygen concentration gradients develop and cause bacterial populations that display different growth rates [20]. The effect of many antibiotics depends on growth. Because most antibiotics aim at some kind of produced macromolecule, it is unexpected that these agents will have much impact on the microorganisms in biofilm that limit macromolecular production, so conventional antibiotics are usually less affected against metabolically inactive or slow-growing cells.

In biofilms, a small subpopulation of bacteria can be reversibly transformed into slowly growing cells. These cells are known as persistent or dormant cells. Persistent cells are generated stochastically or under endogenous stress (e.g., oxidative stress

and exposure to antibiotics) and are highly resistant to being killed by antibiotics [21, 22]. When these cells are compared with active and rapidly growing bacteria, lower metabolism rate makes these cells less susceptible to antibiotics. High levels of persistent cells are seen in chronic urinary tract infections and the lungs of patients with cystic fibrosis, especially, when the penetration of the immune system components is limited. The dormant phenotype is characterized by down-regulation of functions, such as energy production and biosynthesis.

Persistent formation is enhanced by toxin/antitoxin (TA) systems induced by environmental factors or DNA damage. TA systems do the following: (i) inhibition of protein synthesis by phosphorylation of the elongation factor, Ef-Tu (e.g., HipBA), translation inhibition and subsequent tolerance to antibiotics; (ii) expressing the TA modules (e.g., TisB toxin forming an anion channel in the membrane) leading to a decrease in PMF and ATP levels; and (iii) breakdown of mRNA (e.g., RelE and MazF toxins) and inhibition of translation. Prolonged treatment with aminoglycosides and RNA polymerase inhibitor rifampicin may prevent persistent resuscitation with synergistic effects with TA systems [23]. It is suggested that fluoroquinolones can induce TisB toxin by causing DNA damage in *Escherichia coli* [24]. In biofilms, many TA systems are associated with multidrug-tolerant persistent cells. However, this tolerance is limited to specific antibiotics and TA [25].

Bacteria are equipped with a range of stress responses that make them possible to deal with environmental change, such as oxidative stress, unexpected temperature changes, low water activity, deprivation, and DNA damage [26]. These adaptive responses serve to enhance bacterial survivability. Adaptive stress responses can influence antimicrobial susceptibility since these responses impact on many of the same cellular components and processes that are aimed by antimicrobials [27].

Heterogeneity in the biofilm is one of the causes of the stress response [26]. Cells within hypoxic zones have decreased metabolic activity and are in a state like stationary phase [28]. It is known that many of the stress responses result in bacterial cells entering stationary phase.

Nutrient starvation also induces (p)ppGpp production, which mediates a global stress response known as the stringent response. The stringent response and (p)ppGpp signaling contribute to multidrug tolerance in *P. aeruginosa* biofilms. It is shown that ofloxacin, gentamicin, meropenem, and colistin killing increased upon inactivation of the stringent response [29]. Nutrient starvation also induced ofloxacin tolerance in *E. coli* K-12 biofilm through mechanisms dependent on the stringent and SOS response [30].

2.5 Quorum sensing

Despite their self-sufficiency, bacteria interact with neighbors to accomplish collective activities, such as bioluminescence production, biofilm development, and exoenzyme secretion. This cooperation occurs through a mechanism: quorum sensing (QS) [31]. Quorum sensing (QS) is cell-to-cell communication at the molecular level controlled by chemical signaling molecules called autoinducers (AIs) [32]. Due to QS, bacteria can recognize the population density by measuring the accumulation of signaling molecules that are secreted from members of the community. The accumulation of the signal in the extracellular environment is adequate to activate the response only when the population density is high [33].

Recent studies indicate that in many bacterial species, activation of QS happens in the formed biofilm activating the maturation and disassembly of the biofilm. The initial adhesion step seems not suitable for the accumulation of signal molecules. Then, with the next steps, the attached bacteria are divided and form microcolonies, population density rises, and so signal molecules can reach adequate levels

to activate the maturation and disassembly of the biofilm in a coordinate manner (**Figure 1**). The time nutrients and other resources become limited and waste products accumulate, biofilm dispersion is imperative to provide bacteria to escape and colonize new niches [33].

P. aeruginosa harbors two complete AHL circuits, *lasI/lasR* and *rhlI/rhlR*, the *lasI/R* circuit being hierarchically positioned upstream, of the *rhlI/R* circuit. It is reported that *las*-mediated QS inhibits the production of exopolysaccharide, Pel, which builds the biofilm matrix [34].

Another element controlled by QS in *P. aeruginosa* biofilm development is rhamnolipids production [35]. These biosurfactant rhamnolipids caused bacterial detachment of *Pseudomonas* biofilms or even biofilms produced by other microorganisms (*Bordetella bronchiseptica* and *C. albicans*) [36].

In *Staphylococcus aureus*, Agr is a QS regulation system [37]. It was demonstrated in *S. aureus* that a specific class of secreted peptides (phenol-soluble modulins, PSMs) that have surfactant-like properties mediates the main impact of Agr in biofilm dispersion. PSM operons transcription is under strict control by AgrA and agr mutants lack PSM production [38]. Also, it is shown that by analysis of biofilm tridimensional structure with confocal laser scanning microscopy, PSMs impacted the biofilm volume, thickness, roughness, and channel formation.

2.6 Efflux pumps

Efflux pumps are membrane proteins that are related to the export of harmful substances from within the bacterial cell into the external environment. They are found in all species of bacteria, and efflux pump genes can be found in bacterial chromosomes or mobile genetic elements, such as plasmids. A wide array of substrates, such as antibiotics, detergents, dyes, toxins, and waste metabolites are extruded by efflux pumps [39].

There are five known different classes of bacterial efflux pumps, which are the major facilitator superfamily (MF), the small multidrug resistance family (SMR), the ATP-binding cassette family (ABC), the resistance nodulation-division family (RND), and the multidrug and toxic compound extrusion family (MATE) [40]. To carry out the antimicrobial agent flow, the ABC family system hydrolyzes the ATP

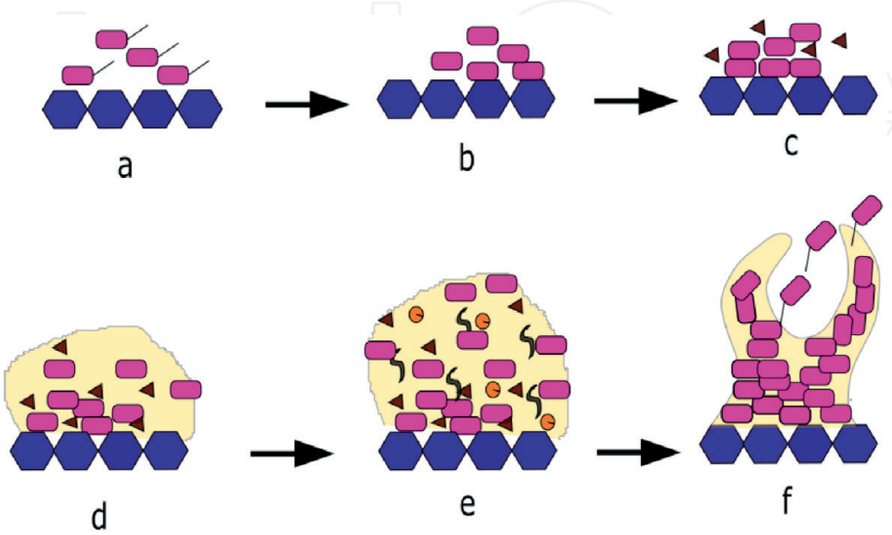

Figure 1.
Stages of bacterial biofilm formation ((a) Planktonic cells adhere to a surface, (b) Initial attachment; cell proliferates to form a monolayer over the surface, (c–d) Increase in cell numbers results in the synthesis of elevated levels of autoinducers and EPS, (e) A mature biofilm with increased resistance to hostile environmental factors, (f) Dispersion of bacteria, ◀: Autoinducers, ʃ: eDNA, ◔: Enzymes).

while the MF family, the MATE family, and the RND family act as secondary carriers and catalyze the drug ion antiproton [41].

Efflux pumps play a role in the natural resistance to antibiotics in some pathogens. These pumps also cause acquired resistance by overexpression and contribute to other resistance mechanisms [20]. Overproduction of the efflux pump can lead to multidrug resistance. Bacterial efflux pumps perform multidrug resistance (MDR) phenotype [42]. The efflux pump slows down the diffusion of hydrophilic solutes by downregulating the "porin" production in several pathogenic bacteria, such as *E. coli*, *Enterobacter aerogenes,* and *Klebsiella pneumonia*, thereby decreasing the transmembrane diffusion of lipophilic solutes [43].

Some multidrug efflux pumps contribute significantly to biofilm formation and this mechanism can be used to help bacteria overcome attacks from several classes of antibiotics. Extremely reduced biofilm formation has been reported for mutant *E. coli* that does not have the various genes associated with efflux pumps [44].

Upregulation of some efflux pumps (MexAB-OprM and MexCD-OprJ) in resistant *P. aeruginosa* biofilms has been observed in the presence of azithromycin [45]. It is reported that flow pump PA 1874-1877 is associated with biofilm-specific resistance to antibiotics. When these genes are mutated, lower resistance to aminoglycosides and fluoroquinolones is seen in biofilm conditions [46].

Efflux pumps may play different roles in biofilm formation; several studies proposed that efflux of EPSs and QS molecules to facilitate biofilm matrix formation and regulate QS, respectively, lead to indirect regulation of genes involved in biofilm formation and influence aggregation by promoting or preventing adhesion to surfaces and other cells [39].

2.7 Genetic diversity

Genetic diversity provides bacterial adaptation, evolution, and survival in hostile environments. Biofilms are considered as a reservoir of genetic diversity. In biofilms, the emergence and spread of antibiotic resistance genes increase with horizontal gene transfer (HGT). HGT can happen through the transfer of plasmids among microorganism cells in a biofilm by conjugation. Actually, studies that are practiced by certain researchers have demonstrated that plasmid moving among bacterial cells might be more effective in biofilms than planktonic cells and that probably arises from proximity of microorganism cells in planktonic shape. In addition, some bacteria have the ability to pick up DNA from the biofilm matrix. The highly hydrated matrix provides favorable conditions for natural transformation [47]. Incidence of antibiotic resistance gene cassettes is determined more than 100-fold higher in biofilms than in planktonic cells [48].

Mutation frequency can be another factor that increases antibiotic resistance or tolerance. There is proof in the literature that cells in biofilms accumulate mutations at a higher rate than planktonic cells and these mutations may contribute to increase of antibiotic resistance [16]. Some bacteria have ability to pick up DNA from the biofilm matrix in addition.

2.8 Multispecies interactions

Many laboratory studies about biofilm associated with antibiotic resistance and tolerance mechanisms have focused on monospecies biofilms in the literature and this issue is becoming progressively apparent. Interactions among microorganisms that are different species in a biofilm can alter the general antimicrobial resistance of the population. When we regard that many infections are polymicrobial, these interactions may be considered clinically important [49].

Studies are showing that antimicrobial resistance in multispecies biofilms is much higher than that in monospecies biofilms in available literature. For instance, it is determined that in vivo *P. aeruginosa* growing in a monospecies biofilm is twice more vulnerable to gentamicin antibiotic than that growing in multispecies biofilm consisting of *S. aureus, Enterococcus faecalis,* and *Finegoldia magna*. The molecular mechanism that underlies this multispecies biofilm model, which increases gentamicin tolerance, is not known [50].

A clinically important model of multispecies biofilm infection includes *Moraxella catarrhalis* and *Streptococcus pneumoniae*. These bacteria play a role in the pathogenesis of otitis media, a biofilm-mediated infection that may be multi microbial. When antibiotic therapy is required, otitis media is commonly treated by amoxicillin. However, in stubborn cases, second-line treatments, such as amoxicillin-associated β-lactamase inhibitor or azithromycin, are applied. It is determined that in the biofilm consisting of two species, *M. catarrhalis* produces β-lactamase that provides resistance of *S. pneumoniae* against amoxicillin. Reciprocally, *S. pneumoniae* protects *M. catarrhalis* from azithromycin with an unknown mechanism [51].

Interactions between different microorganisms and their effects on biofilm susceptibility to antibiotics have also been examined in polymicrobial biofilms. *C. albicans*, an opportunistic fungal pathogen, and *S. aureus* have a high resistance to vancomycin in a dual species. In a biofilm that is composed of *C. albicans* and *S. aureus*, *S. aureus* is associated with the fungal hyphae via the *C. albicans* Als3p adhesin and becomes covered with biofilm matrix probably derived from *C. albicans* [52].

Owing to the fungal matrix component, β-1,3-glucan, which is thought to act as a barrier to vancomycin diffusion into the biofilm, Staphylococcal resistance to vancomycin is increased in polymicrobial biofilms formed with *C. albicans* [53].

In polymicrobial biofilms, molecular basis may increase antibiotic resistance. In a study focused on *P. aeruginosa* and *S. maltophilia* two-species biofilm, it is determined that an intercellular signaling molecule that is secreted by *S. maltophilia* is sensed by the two-component sensor, BptS in *P. aeruginosa*, inducing upregulation of the PmrA-regulated PA3552_3559 and PA4773_4775 genes. These two operon gene products provide resistance to polymyxins, which is a cationic antimicrobial peptide. Actually, *P. aeruginosa* cultured in a biofilm with *S. maltophilia* have reduced vulnerability to polymyxin B and colistin compared to *P. aeruginosa,* single-species biofilms [54].

3. Approaches aimed at overcoming biofilm resistance

Biofilm infections can be treated and dispersed by the mixture of traditional antibiotics and substances called biofilm disrupting. The dissolution of biofilm is the first step in the ability of the host organism's immune system to remove microbial pathogens [55]. The combined antibiotics with the biofilm-dispersing medicines can bring a promising outcome. Most biofilm-dispersing medicines do not kill the pathogenic cells when they are used alone. For instance, patulin was analyzed with the aim of acyl-homoserine lactone removal in *P. aeruginosa*, but it had no effect on the existence of *P. aeruginosa* cells in a given biofilm. Although only patulin had no effect on the *P. aeruginosa,* the combination of patulin with antibiotic tobramycin was more effective and caused serious killing of the bacterial cells [56]. Another study showed that the mixture of the quorum controlling compounds with the antibiotic tigecycline increased the susceptibility of *S. aureus* fourfold compared to tigecycline alone [57]. Furthermore, the treatment of *S. aureus* with the mixture of cis-2-decenoic acid and ciprofloxacin is improved from 11 to 87% compared to antibiotic alone.

Considering the rising number of antibiotic-resistant pathogens, QS inhibitors can be used as a mixture with the remaining sensitive antibiotics to complement their effects. These molecules mainly act by suppressing the QS system, and their practice with antibiotics leads to effective cure at much lower dosages of the drug than necessary, which may result in reduced therapeutic costs. These combinations can be beneficial in the cure of chronic infections, such as chronic urinary tract, cystic fibrosis, or prosthetic infections and biofilms are a barrier to antibiotic diffusion in these chronic diseases.

There is an urgent need for new methods in the cure of biofilm-associated infections. For instance, cyclic di-GMP (c-di-GMP) is a commonly protected prokaryotic second messenger signal molecule necessary for biofilm development [58]. New inhibitors of diguanylate cyclase enzymes were identified by using in silico screening, and they tested them successfully in vitro. Inhibitors of flow pumps can also be recommended to complement the effect of antimicrobial agent and needed to be tested in vivo.

The choice of antimicrobial agents also seems to be significant because some of them may act as agonists for biofilm formation and some may disrupt it. The usage and dosages of novel antibiotics should be checked and clinically synthesized antibiotics should be tested at impactful concentrations by considering their distribution in biofilms and the detrimental effects of signaling molecules. Other compounds act as key enzymes in the biosynthesis of these signaling molecules and play a role in regulating virulence factor production and biofilm formation. A ligand-based strategy will allow the identification of new inhibitors in the future.

Better usage of the new active molecules can be supported by understanding mechanisms of antimicrobial agents activity as well as the molecular mechanisms associated with biofilm formation and recalcitrance [5].

4. Conclusion

Biofilm infections are highly resistant to antibiotics and physical treatments and it is known that there are many strategies that support biofilm antibiotic resistance and tolerance, such as persistent cells, adaptive responses, and limited antibiotic penetration. It is also known that the underlying mechanisms of antibiotic tolerance and resistance in biofilms have a genetic basis in many cases.

In human diseases, highly organized bacterial cells gradually induce immune responses to form biofilms responsible for chronic infections that lead to tissue damage and permanent pathology. Therefore, the formation of biofilm is considered a critical concern in health care services.

Exploring promising cure methods for biofilm-associated infections is an urgent task. Few innovative and effective antibiotic strategies have been tried, such as dispersion of biofilms, antibiotic combinations with quorum sensing inhibitors, and a mixture of all these new techniques. Although the mentioned anti-biofilm strategies are important research areas, they are still in infancy and have not undergone clinical research and entered the commercial market. We hope that new anti-biofilm molecules based on finding universal substances that do not harm cells and synergistic with commonly used antibiotics will be available in the near future.

Conflict of interest

The authors declare no conflict of interest.

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