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Biofilm-Related Infections: Bridging the Gap between Clinical Management and Fundamental Aspects of Recalcitrance toward Antibiotics

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SUMMARY

Surface-associated microbial communities, called biofilms, are present in all environments. Although biofilms play an important positive role in a variety of ecosystems, they also have many negative effects, including biofilm-related infections in medical settings. The ability of pathogenic biofilms to survive in the presence of high concentrations of antibiotics is called "recalcitrance" and is a characteristic property of the biofilm lifestyle, leading to treatment failure and infection recurrence. This review presents our current understanding of the molecular mechanisms of biofilm recalcitrance toward antibiotics and describes how recent progress has improved our capacity to design original and efficient strategies to prevent or eradicate biofilm-related infections.

INTRODUCTION

For centuries, humankind suffered from acute bacterial infections and life-threatening diseases caused by pathogens such as *Streptococcus pneumoniae*, *Vibrio cholerae*, and *Yersinia pestis*. The discovery and use of hygiene, antibiotics, and vaccines led to massive reductions in the burden of and mortality related to such infections, mainly caused by individualized pathogenic bacteria (1, 2). Following this "antibiotic golden age," physicians confronted two major challenges: the occurrence and spread of antibiotic-resistant bacteria and the rise of chronic, difficult-to-eradicate infections (3). Indeed, D. Holsclaw, referring to cystic fibrosis (CF) patients in 1980, stated that "even with the use of large doses of parental antibiotics, *Pseudomonas* cannot be eradicated from the sputum" (4).

Meanwhile, environmental microbiologists have progressively established that surface-associated bacterial biofilm communities are widespread in all types of natural environments, where they often prevail, in contrast to individualized, planktonic bacteria (5–8). While biofilms display specific biological properties compared with planktonic bacteria, N. Høiby, J. W. Costerton, and their collaborators were the first to suspect a direct correlation between development of biofilms and persistent infections, notably in the case of *Pseudomonas aeruginosa* colonizing the lungs of CF patients (9, 10). Consistently, the decades that followed confirmed the role of biofilms in the pathophysiology of tissue-related infections (Fig. 1) (11). Furthermore, it was recognized that the widespread use of various types of indwelling medical devices implanted in humans could favor microorganism adhesion and cause colonization, leading to infection. In this regard, the first evidence of the involvement of biofilms in device-related infections was provided in 1982 by an electron microscopy study of a pacemaker lead in a patient with recurrent *Staphylococcus aureus* bloodstream infection (BSI) (12). Since then, almost all types of indwelling devices have been associated with the occurrence of bacterial or fungal biofilms (Fig. 1) (2).

Due to their high tolerance toward antibiotics, these chronic tissue-related and device-related infections are difficult to treat and expose the patient to the risk of recurrence (13, 14). During a biofilm-related infection, planktonic bacteria originating from the biofilm can spread into the bloodstream or around the source of the infection (13, 14). Whereas planktonic bacteria can be eradicated via the combined action of antimicrobials and cellular and humoral host immune responses, a subset of highly tolerant biofilm bacteria frequently survive the treatment and can cause infection recurrence. In most cases, removal of the colonized device or surgical excision of infected tissue is the only efficient way to eradicate a biofilm-related infection (1, 13). Hence, the ability of biofilm bacteria to withstand antibiotics significantly influences the outcome and management of patients (1).

This review provides a description of the mechanisms involved in the capacity of bacterial biofilms to survive in the presence of antibiotics and presents recent therapeutic approaches developed to specifically target biofilm-related infections.

MECHANISMS OF BIOFILM RECALCITRANCE TOWARD ANTIBIOTICS

Once a biofilm is established, bacteria are able to survive after various types of physicochemical aggression, including UV light, heavy metals, acidity, changes in hydration or salinity, and phagocytosis (15–19). In addition, biofilm bacteria also display a characteristic ability to withstand antibiotic-mediated killing, which is directly responsible for a significant number of therapeutic difficulties encountered in clinical settings.

It is now clear that well-studied mechanisms involved in classical antibiotic resistance, such as efflux or antibiotic-modifying enzymes, play only a marginal role in the ability of biofilms to survive antibiotics (20, 21). Indeed, bacteria embedded in a biofilm are able to partly withstand high concentrations of bactericidal antibiotics even when these bacteria are fully susceptible to such antibiotics *in vitro* under planktonic conditions (22). This phenomenon, here named "recalcitrance of biofilm bacteria toward antibiotics," is complex and is due to several tolerance and resistance mechanisms, as described below.

Tolerance and Resistance: Biofilm Recalcitrance Defined

The study of how *in vitro* planktonic bacteria escape antibiotic treatment led to the definition of two different concepts: resistance and tolerance.

Resistance: how to grow in the presence of an antibiotic. Resistance can be defined as the ability of a microorganism to multiply in the presence of a toxic compound (antibiotic or antiseptic) and can be applied to both bacteriostatic and bactericidal antibiotics (14, 23–25). Resistance is usually tested by measuring the MIC of a compound, i.e., the lowest concentration inhibiting

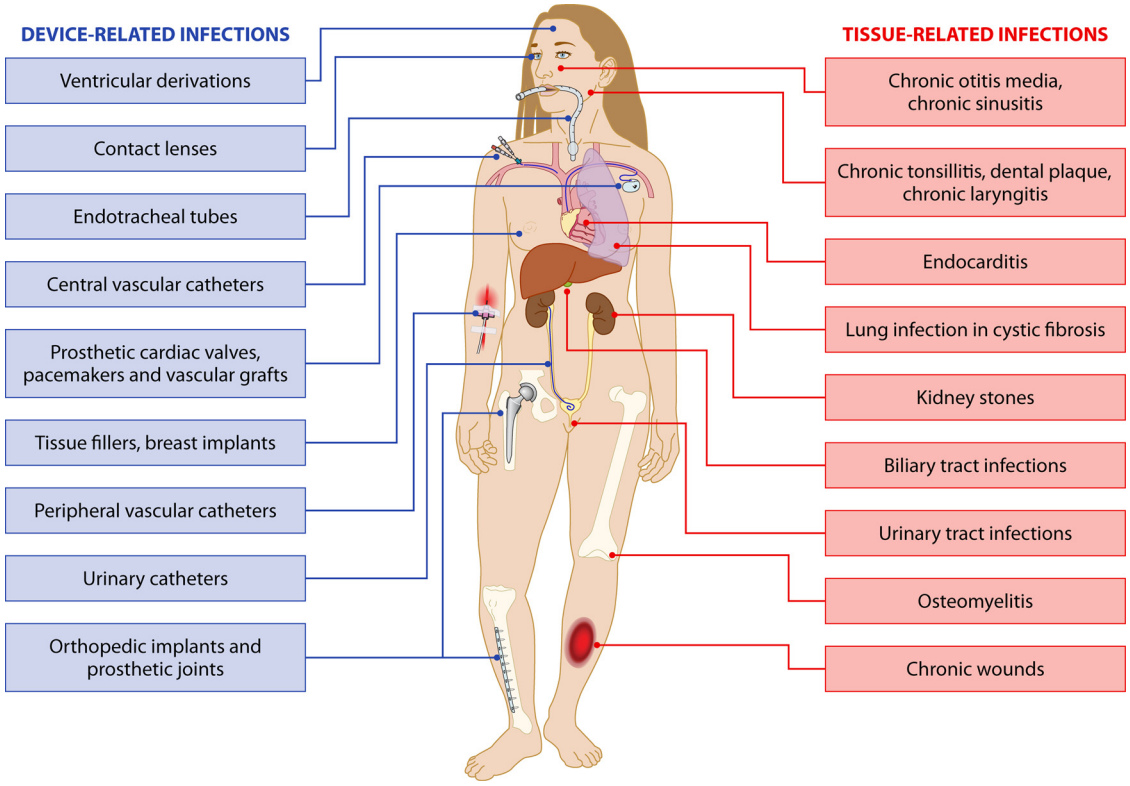

FIG 1 Biofilm-related infections. (Adapted from reference 365 with permission of the publisher and from reference 11.)

growth of a standardized inoculum of exponentially growing bacteria. A bacterium is more resistant toward an antibiotic if its MIC is higher than that of another bacterial strain. Numerous mechanisms explain this phenomenon, including antibiotic efflux, reduced permeability to antibiotics, activities of enzymes that modify or destroy antibiotics, and modification of the antibiotic target (through mutation, enzymatic action, or the presence of an alternate target) (21). Generally speaking, these resistance mechanisms avoid interactions between the antibiotic and its target, thereby allowing bacteria to multiply in the presence of the antibiotic (13). Resistance is often genetically inherited and therefore transmitted from mother to daughter bacteria, or it can be acquired through horizontal gene transfer.

Tolerance: how to avoid antibiotic-induced cell death. In contrast to resistance, tolerance can only be associated with the use of bactericidal antibiotics, i.e., an antibiotic able to kill at least 99.9% of a bacterial population after overnight incubation (14). The lowest antibiotic concentration that enables reaching this threshold is called the minimal bactericidal concentration (MBC), according to the Clinical and Laboratory Standards Institute (CLSI) (14).

Thus, tolerance can be defined as the absence of growth but the existence of bacterial survival in the presence of a bactericidal antibiotic (13, 26, 27). Hence, a tolerant bacterial strain may be susceptible to a bactericidal antibiotic, as indicated by an unmodified MIC, while at the same time displaying increased survival, as defined by an MBC/MIC ratio of ≥32 or a kill rate of ≤99.9% after a 24-h challenge (28).

Two types of tolerance have been described: genotypic and phenotypic. In the first case, the presence of a genetic modification leads to a reduced ability of the antibiotic to kill the bacteria and can be transmitted to daughter cells. Examples have been described for *Streptococcus pneumoniae* and for small-colony variants (SCV) of *Staphylococcus aureus* (26, 29). In the case of phenotypic tolerance, the environment is not favorable to the action of antibiotics, thus leading to a decreased ability to kill. This is the case for nongrowing bacteria that are tolerant to β-lactams despite a normal MIC. Phenotypic tolerance is therefore rapidly reversible after the return to a growth-promoting medium (28).

Biofilm recalcitrance: a problematic mixture of resistance and tolerance. In the study of biofilms, bacterial survival is often determined after an antibiotic challenge. This phenotype is therefore closer to the definition of tolerance than to that of resistance, as biofilm bacteria do not grow but a subset of them is able to survive in the presence of high concentrations of bactericidal antibiotics (up to 1,000× MIC) (14, 22, 27, 30). However, we will see that in addition to tolerance, resistance mechanisms *sensu stricto* also contribute to biofilm survival against antibiotics. Therefore, neither of these two concepts fully applies to biofilms. Recalcitrance, on the other hand, was previously proposed to characterize the capacity of biofilm bacteria to withstand treatment (31, 32). Since the word "recalcitrance" covers the notion of nonsusceptibility to (antimicrobial) control of refractory biofilms, we use it here to characterize the ability of a subset of biofilm bacteria to survive in the presence of antibiotics. Biofilm recalcitrance is reversible and mainly noninherited, and it disappears when the biofilm is disrupted and bacteria return to a planktonic state (20, 33).

Biofilm Recalcitrance Is Multifactorial

Recent studies on the ability of bacterial biofilms to survive high concentrations of antibiotics led to a complete shift in our under-

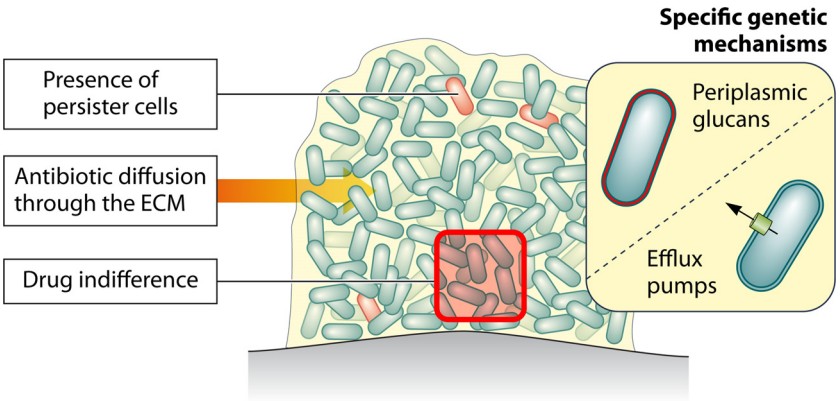

FIG 2 Summary of the main mechanisms involved in recalcitrance of biofilms toward antibiotics. (Adapted from reference 366 with permission of the publisher.)

standing of mechanisms involved in biofilm recalcitrance. This phenomenon is multifactorial and, depending on the class of antibiotic used, involves different mechanisms, including impaired antibiotic diffusion, drug indifference, expression of biofilm-specific genetic mechanisms, and the presence of persister cells (Fig. 2).

Antibiotic penetration. Historically, it was proposed that the extracellular matrix (ECM) surrounding bacteria was responsible for biofilm recalcitrance. Many reports suggested that mechanical and physicochemical properties of the biofilm matrix can reduce or delay penetration of numerous compounds, including antibiotics and antiseptics (34, 35). For instance, the effect of an antibiotic can be reduced after adsorption on the matrix due to electrical interactions with polymers surrounding biofilm bacteria (20, 36). Other studies reported slow penetration of positively charged aminoglycosides through negatively charged polymers of the biofilm matrix (37, 38). In this regard, the chemical structure of the biofilm matrix is important, and it has been shown that even for a single pathogen, different types of exopolysaccharides can be involved, depending on the environment surrounding the biofilm (39). Monitoring of antibiotic diffusion through cardiac vegetation in an *in vivo* model of endocarditis demonstrated that a diffusion gradient could be observed in the case of teicoplanin (a glycopeptide) and penicillin (40). In contrast, tobramycin was shown to be homogeneously distributed. Conversely, in the case of *P. aeruginosa* biofilm, tobramycin was shown to exhibit delayed and reduced diffusion *in vitro* (41, 42). Thus, experimental data regarding the diffusion of an antibiotic through the biofilm matrix cannot be extrapolated to another bacterial strain and should be interpreted carefully (Table 1).

The study of chlorine antiseptic diffusion by use of microelectrodes showed that the chlorine concentration in the bulk of a mixed biofilm (*P. aeruginosa* and *Klebsiella pneumoniae*) represented only 20% of the concentration in the bulk liquid after 2 h (43). These results were confirmed using bacteria entrapped in alginate beads, with a time to reach 50% of the chlorine bulk concentration at the bead center of approximately 46 h (44).

However, many reports also suggested that reduction of antibiotic penetration cannot fully explain biofilm recalcitrance toward antibiotics. Indeed, antibiotics such as fluoroquinolones, rifampin, and ampicillin penetrate well through the matrix, even though they fail to eradicate 100% of biofilm bacteria (22, 42, 45,

TABLE 1 Penetration of antibiotics through thebiofilm extracellular matrix

Microorganism	Antibiotic	Penetration	Reference(s)
P. aeruginosa	Piperacillin	Reduced/yes	367, 60
	Imipenem	Yes	60
	Ofloxacin	Yes	60, 368
	Ciprofloxacin	Yes	45, 60, 369, 370, 42
	Levofloxacin	Yes	60, 369, 370
	Sparfloxacin	Yes	60
	Gentamicin	Reduced	60
	Amikacin	Reduced	60
	Tobramycin	Reduced	41, 42
	Amoxicillin-clavulanic acid	Yes	45
	Fosfomycin	Yes	45
	Clarithromycin	Yes	368
E. coli	Moxalactam	Yes	371
	Fosfomycin	Yes	45
	Amoxicillin-clavulanic acid	Yes	45
	Ciprofloxacin	Yes	45
K. pneumoniae	Ampicillin	No	22
	Ciprofloxacin	Yes	22
S. epidermidis	Rifampin	Yes	372, 46
	Vancomycin	Yes	373, 372
	Ciprofloxacin	Yes	374
	Ofloxacin	Yes	368
	Clarithromycin	Yes	368
	Daptomycin	Yes	228
	Cefotaxime	Reduced	374
	Oxacillin	Reduced	374
	Cefotiam	Yes	368
	Amikacin	Yes	374
S. aureus	Vancomycin	Yes/reduced	48, 374
	Cefotaxime	Reduced	374
	Oxacillin	Reduced	374
	Ciprofloxacin	Yes	374
	Amikacin	Yes	374

46). Moreover, even in the case of compounds slowly diffusing within biofilms, most antibiotics ultimately reach all biofilm bacteria. For instance, *P. aeruginosa* and *Escherichia coli* 24-h *in vitro* biofilms were not eradicated by a 24-h treatment with fosfomycin or ciprofloxacin, whereas these drugs reached more than 50% of the bulk concentration within 6 h (45). The same observation was made concerning *K. pneumoniae* biofilms and ciprofloxacin (22). Studies using fluorescent tetracycline demonstrated that 2-day biofilms were less susceptible than planktonic bacteria, whereas tetracycline-mediated fluorescence was present throughout the biofilm within 10 min (47).

On the other hand, delayed antibiotic penetration may have important phenotypic consequences. For instance, bacterial cell physiology could adapt to the presence of antibiotics though metabolic or transcriptional adaptation induced by antibiotic stress (48). Furthermore, due to slow diffusion, biofilm bacteria could be transiently exposed to subinhibitory concentrations of antibiotics (see below). Limited diffusion can also protect biofilms from degradable antimicrobials. Indeed, *P. aeruginosa* produces AmpC β-lactamase, and it has been demonstrated that 2.5% of clinical isolates from CF patient sputa are totally derepressed, with a high basal level of enzyme production that can be increased further through β-lactam-mediated induction and β-lactamase accumulation in the biofilm matrix (49, 50). In clinical samples, insertion sequences inactivating the *ampD* gene have been described for CF patients with constitutively high expression of β-lactamase (51). The association of a diffusion barrier that slows down diffusion of β-lactams and the presence of a hydrolyzing enzyme may act synergistically, especially if the enzymes degrade antibiotics faster than they diffuse (52–54).

Drug indifference and an altered microenvironment. Deep biofilm layers correspond to a particular physicochemical microenvironment due to various gradients of nutrients, waste, pH, oxygen, and metabolic by-products through the ECM (55). Since many antibiotics are more active against bacteria that are metabolically active and growing, the characteristic lack of nutrients or anoxia of these microenvironments can antagonize the effects of antibiotics (56–58). This is the case for β-lactam antibiotics, which target the bacterial membrane and are effective only against actively growing bacteria undergoing cell division (14, 59). Consistently, the effects of β-lactams against *P. aeruginosa* biofilms have been correlated with the metabolic activity of biofilm bacteria (60, 61). Similarly, the low oxygen concentrations found in deep layers of *P. aeruginosa* biofilms reduce tobramycin and ciprofloxacin bactericidal effects (42). Other physicochemical characteristics can impair the effects of antibiotics, such as low pH and the anaerobic environment, leading to decreased activities of aminoglycosides (20, 62–64).

However, a reduction in β-lactams or in aminoglycoside antibiotic efficacy in altered microenvironments does not fully account for the observed biofilm recalcitrance toward antibiotics that are active against nongrowing bacteria, such as fluoroquinolones (61, 65, 66).

Contributions of genetically determined mechanisms. Many investigators have tried to identify genetic mechanisms of recalcitrance specifically activated during the biofilm lifestyle. To do so, Mah and collaborators used a random transposon insertion library screened for *P. aeruginosa* mutants, making biofilms more sensitive to tobramycin, and they identified 3 genes or operons: *ndvB*, PA1875 to PA1877 (PA1875–PA1877), and *tssC1* (67). The first gene identified, *ndvB*, encodes a putative glucosyltransferase that was later shown to be required for synthesis of cyclic-β-(1,3)-glucans (68). Periplasmic glucans interact with and sequester aminoglycosides in the periplasm and keep them away from their intracellular target. Note that these glucans are also secreted into the biofilm matrix. An *ndvB* mutant was also more sensitive to ciprofloxacin, ofloxacin, gentamicin, and chloramphenicol, suggesting that induction of *ndvB* was also involved in recalcitrance toward other classes of antibiotics. In that case, periplasmic glucans inhibit the interaction between an antibiotic and its target. Interestingly, *ndvB* seemed also to contribute to *P. aeruginosa* antibiotic tolerance by an unknown mechanism involving increased ethanol oxidation (69), therefore suggesting that the *ndvB* action is the sum of pleiotropic effects. The second locus identified, PA1875–PA1877, corresponds to a 3-gene operon coding for an outer membrane protein, an ATP binding cassette transporter, and a membrane fusion protein (70). Deletion of these genes resulted in biofilms with increased sensitivity to tobramycin and ciprofloxacin. Moreover, a deletion mutant accumulated more tobramycin than the wild type, suggesting that the identified locus could code for an efflux pump. The last identified gene, *tssC1*, was shown to be an essential component of the type VI secretion (T6S) system potentially involved in cell-to-cell interactions (71). More recently, three other loci, i.e., PA0756-PA0757 (encoding a putative two-component regulatory system), PA2070, and PA5033 (both encoding hypothetical proteins of unknown function), were shown to contribute to the biofilm-specific antibiotic tolerance of *P. aeruginosa* (72).

Several studies performed in *P. aeruginosa* and *E. coli* suggested that efflux pumps, induced specifically under biofilm conditions and removing antibiotics from the bacterial intracytoplasmic space, could be involved in biofilm-specific recalcitrance. In *P. aeruginosa* biofilms, MexAB-OprM and MexCD-OprJ pumps are involved in the efflux of azithromycin, colistin, and ofloxacin, but in the latter case, only at low concentrations (73–75). Interestingly, MexEF-OprN and MexXY-OprM are upregulated in response to reactive oxygen species (ROS) and could help in removing cellular elements damaged by ROS (76). From the sputa of CF patients, highly tolerant *P. aeruginosa* strains with mutations in the *mexZ* repressor controlling expression of the MexXY-OprM pump have been isolated (77). Strikingly, deletions in the *mexXY* locus restored wild-type resistance but did not affect antibiotic tolerance, suggesting that MexXY-OprM plays only a marginal role in biofilm recalcitrance (77). Recently, the DNA-binding transcriptional regulator BrlR was shown to contribute to *P. aeruginosa* tolerance (78). When inactivated, biofilm bacteria are more susceptible to hydrogen peroxide and antibiotics of different classes, including tobramycin and norfloxacin. On the other hand, *brlR* overexpression increased *P. aeruginosa* tolerance toward antimicrobials. The same group identified BrlR as an activator of *mexAB-oprM* and *mexEF-oprN* (79) and the two-component hybrid protein SagS as a possible upstream regulator of BrlR (80). Recently, SagS was shown to contribute to BrlR activation and tolerance toward antibiotics through an increase of the level of the second messenger cyclic di-GMP (c-di-GMP) (81).

In *E. coli*, many efflux pumps, such as AcrAB-TolC, are upregulated in biofilms and may remove toxic compounds, including antibiotics (82). High-thoughput screening of *E. coli* mutants showed that *rapA* mutants displayed decreased resistance toward penicillin, norfloxacin, chloramphenicol, and gentamicin (83). It

was demonstrated that *rapA* not only regulates *yhcQ*, a gene encoding a putative multidrug resistance (MDR) pump, but also *yeeZ*, a gene suspected to be involved in ECM production (83). Thus, a dual *rapA*-mediated action was proposed, with efflux through a pump and reduced penetration through an increase in polysaccharide production (83).

The hypothesis of biofilm-induced *E. coli* or *P. aeruginosa* efflux pumps preventing antibiotic action is attractive. However, these pumps play a role mostly at low antibiotic concentrations, and, to date, the involvement of biofilm-specific efflux pumps in biofilm recalcitrance has remained controversial (14, 70, 73, 74).

Bacterial persistence. While the above-mentioned mechanisms play an important role in the inability of antibiotics to fully eradicate biofilm bacteria, they cannot fully explain biofilm recalcitrance. This is particularly clear in the case of fluoroquinolones: although these antibiotics are able to kill nondividing cells and diffuse easily throughout the biofilm matrix, experimental studies have demonstrated their inability to fully eradicate biofilms (27). The presence of an isogenic subpopulation of tolerant bacteria, called "persister cells" or "persisters," is now considered to explain most of the biofilm recalcitrance toward antibiotics (31). The presence of persisters in the bacterial population has been known since the origin of the antibiotic era; indeed, Joseph Bigger identified them in a population of *S. aureus* planktonic bacteria 70 years ago. When Bigger analyzed what he called "variations in sterilizing power" by quantifying the precise number of surviving bacteria after 3 days of treatment, he recovered fewer than 100 surviving individuals from an initial population of 250,000,000 bacteria (0.00004% survival) (84). He then showed that this subpopulation of bacterial cells resumed growth after the end of antibiotic exposure and that they were not resistant mutants, since they exhibited the same survival pattern following another exposure to antibiotics. He concluded that "the only hypothesis which seems to explain the occurrence of a small number of survivors out of the millions of cocci originally present is that these differ from the majority of their fellows in that they are capable of surviving a concentration of penicillin which, in the time or action allowed, kills the others" (84). He called these survivors "persisters" and suggested that they were in a dormant and nondividing phase and that their production was not due to penicillin. Retrospectively, he described most of what constitutes our current knowledge of persisters (Fig. 3): (i) persisters make up less than 1% of a bacterial population and are equally present in late-stationary-phase cultures and biofilms; (ii) they do not multiply in the presence of antibiotics, and their phenotypic tolerance is not related to any genetic modifications but rather to a phenotypic state; (iii) they are isogenic toward nonpersisters; and (iv) once persisters resume growth, they display the original, nonpersister antibiotic tolerance profile.

At the population level, the presence of persisters can be viewed as an insurance strategy (85). In case of intense stress for the community, persisters may survive and permit the survival of the community. It has also been proposed that persisters might be bacteria that escape antibiotic-induced programmed cell death (PCD). In that case, the antibiotic is able to interact with its target, thereby leading to growth inhibition; on the other hand, bacteria do not die because of inactivation of PCD (25).

Analysis of survival curves of antibiotic-treated biofilms suggested the presence of a subpopulation of tolerant bacteria surviving bactericidal antibiotics despite increased concentrations and times of exposure, leading to the hypothesis that persisters may play a part in biofilm recalcitrance toward antibiotics (73). Indeed, when exposed to increasing concentrations of fluoroquinolones (active against nondividing cells and without any diffusion impairment), biofilm bacteria are killed until reaching a survival plateau, thereby creating a biphasic curve such as those seen under planktonic conditions (Fig. 3). These tolerant bacteria are mostly persisters, and many *in vitro*, *in vivo*, and clinical studies support the idea that they are responsible for most of the antibiotic recalcitrance of biofilms.

Persisters Play a Central Role in Biofilm Recalcitrance toward Antibiotics

Persisters and clinical issues. Several *in vitro* and *in vivo* studies demonstrated the presence of highly tolerant bacterial persisters in biofilms formed by Gram-positive and Gram-negative pathogens (86–89). Clinical demonstration of the presence of persisters can be inferred from the risk of infection recurrence during biofilm-related infections. For instance, in the case of catheter-related bloodstream infection (CRBSI), even after local treatment with high concentrations of antibiotics (up to 1,000-fold higher than the MIC) for 2 weeks, more than 20% of infections relapse because of the survival of persisters inside the biofilm (90). Thus, the currently proposed model to explain biofilm recalcitrance toward antibiotics relies mainly on the presence of persister cells (13, 14). For antibiotics such as fluoroquinolones, which freely diffuse through the matrix and kill nondividing bacteria, impaired antibiotic diffusion, drug indifference, and specific genetic mechanisms play minor roles in biofilm recalcitrance. Conversely, persisters are able to survive antibiotic-mediated bacterial cell death induced by any bactericidal antibiotics. Furthermore, persisters inside the biofilm matrix escape the effect of the host immune system. Once antibiotic treatment is withdrawn, persisters hiding in the matrix can resume growth, repopulate the biofilm, and cause infection recurrence.

The main mechanisms involved in persister generation. Because persisters are isogenic and present prior to the introduction of antibiotics, they are now believed to appear through a phenotypic switch (91). Several factors and mechanisms have been described as playing important roles in the occurrence of this switch. Most studies on the molecular mechanisms involved in persister formation were conducted with planktonic rather than biofilm bacteria. It is now believed that the presence of persisters is related to both passive and active mechanisms, environmental factors, and stochastic gene expression.

(i) Contribution of dormancy to bacterial persistence. Dormancy can be defined as a state of low metabolic activity during which bacteria do not proliferate without a resuscitation phase (92). Therefore, truly dormant cells do not display metabolic activity. Different lines of argument suggest a link between dormancy and persistence. By use of microfluid devices, *E. coli* persisters were shown to be nongrowing before the introduction of antibiotic (91). Furthermore, using an unstable fluorescent reporter gene associated with a ribosomal promoter (*rrnB*P1, which controls expression of *rrnB* genes expressed at high levels during growth), it was shown that a weakly fluorescent population (i.e., with low ribosomal activity) was enriched in persisters (93). Even when enrichment is significant, it is important that not all dormant bacteria are persisters; conversely, all persisters do not necessarily correspond to dormant cells (93).

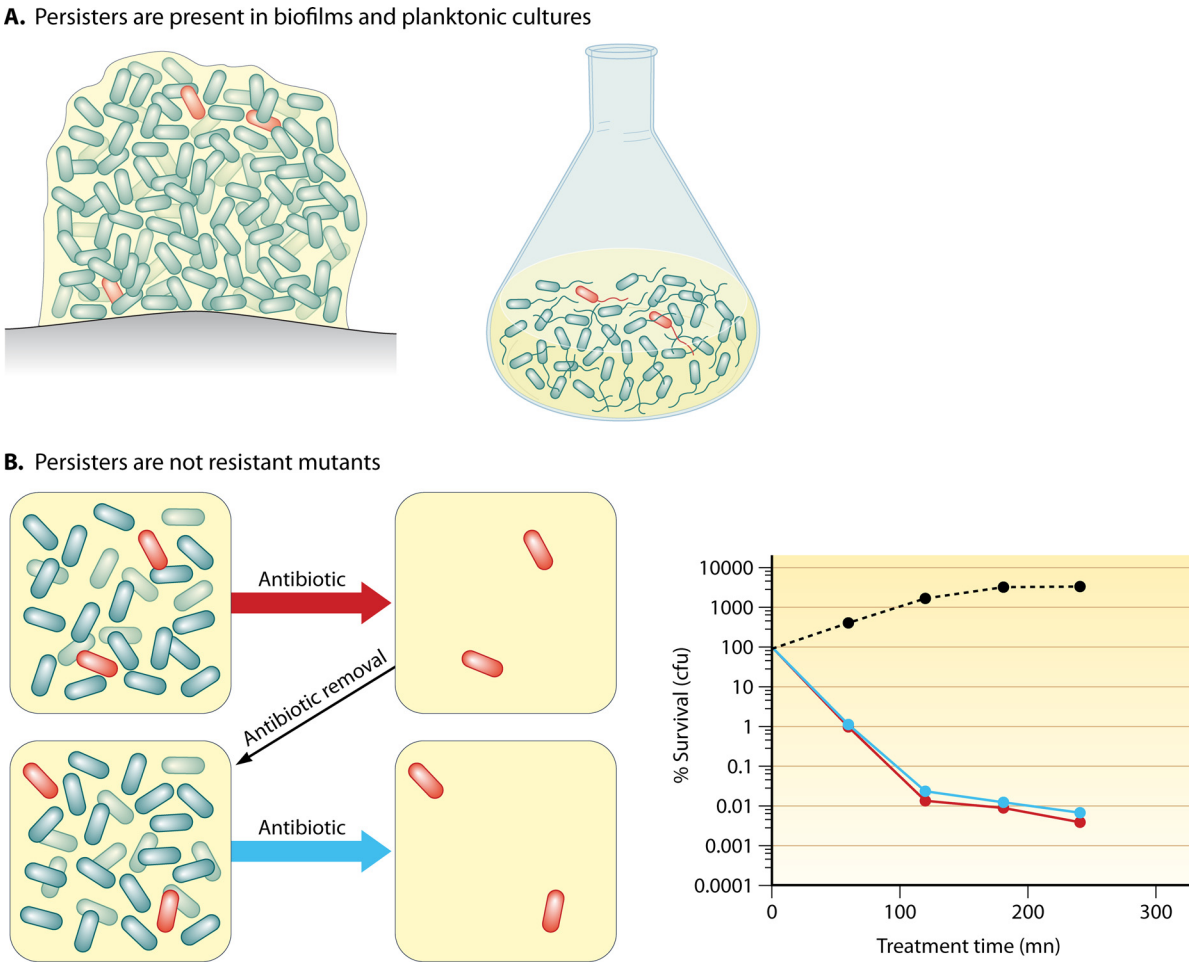

FIG 3 Main phenotypic characteristics of persister cells. (A) Persisters (red bacteria) are present under planktonic and biofilm conditions and account for only a small subset of the whole population (0.001% to 0.1%). (B) Persisters are not resistant mutants. After treatment of a bacterial population with a bactericidal antibiotic, all nonpersister cells die, giving a biphasic survival curve. After a rapid decrease, surviving cell fractions reach a plateau corresponding to persisters (red curve). After antibiotic removal and addition of rich medium, persisters resume growth. The population obtained displays a susceptible phenotype toward the antibiotic (blue curve). If a resistant mutant were present, it would be able to grow in the presence of the antibiotic (dotted line). Panel B was inspired by previous reports (13, 31, 94).

Therefore, it is likely that passive dormancy *per se* is not entirely responsible for the persister phenotype (94). A recent study confirmed these findings by using flow cytometry sorting of *E. coli* cells based on their level of metabolic activity and/or cell division (95). The authors showed that bacteria that grow rapidly prior to antibiotic exposure can give rise to persisters, whereas low metabolic activity or a low growth rate only increases the odds of entry into persistence (95).

(ii) TA modules. Expression of toxin-antitoxin (TA) modules often leads to a shutdown of bacterial cellular processes. Although the molecular nature of TA modules varies, from protein to RNA molecules, the toxin is usually a stable component that inhibits major cellular functions, such as translation and replication (96, 97). To keep a toxin in check, degradable antitoxin antagonizes the effect of the toxin through formation of an inactive complex. In the case of a TA module carried by a plasmid, after cell division, newly formed daughter cells die unless receiving the plasmid, as the antitoxin will be degraded through proteolysis, allowing the toxin to exert its deleterious effects in plasmid-free bacteria. Such a system allows maintenance of the plasmid and was previously referred to as an "addiction module" (98). In *E. coli*, at least 36 putative TA modules have been identified (96, 99). Since toxins halt growth and thus reduce the activity of the antibiotic target, they appear to be attractive effectors of the switch to the persister state (100, 101).

In *E. coli*, the first TA locus associated with an increased level of persister production was *hip* (for "high persister"), identified through random mutagenesis. In an *hipA7* mutant, the persister level is increased 1,000-fold compared to that of the wild type, with increased tolerance toward β-lactams, fluoroquinolones, and aminoglycosides (102, 103). This *hipA7* allele is associated with two point mutations resulting in a gain of function. As overexpression of HipA is toxic and leads to the arrest of cell division, it has been proposed that the locus carries a toxin-antitoxin module (104). The deletion of *hipB* is lethal because of HipA toxicity, suggesting that HipB is the repressor of the operon (105). Note that deletion of the complete *hip* locus has no effect on persister frequency in exponentially growing bacteria, possibly because of TA module redundancy (94, 105). Another explanation is that the HipBA module contributes to the persister switch only in cases of

slow growth (stationary-phase cultures) (106). HipA was first thought to phosphorylate the translation factor EF-Tu, leading to persistence via cell stasis (107). However, it was recently shown that HipA more likely inhibits glutamyl-tRNA synthetase (GltX) through phosphorylation and thus triggers the synthesis of ppGpp (see below) (108).

Using an *hipA7 E. coli* mutant, the gene expression profile of persisters after lysis of nonpersisters by β-lactams demonstrated overexpression of genes coding for TA modules (*dinJ*/*yafQ*, *yefM*, *relBE*, and *mazEF*) and for proteins blocking critical cellular functions (Rmf, which inhibits translation; UmuDC, which inhibits replication; and SulA, which inhibits septation) (103). The study confirmed that overexpression of *relE* led to growth inhibition and increased the level of persisters. Note that, in *E. coli*, the MazEF chromosomal TA module and the RelE toxin are known to induce reversible stasis because of inhibition of translation and/or replication (109). Deletion of the *hipBA* locus leads to a decrease in the level of persisters in stationary-phase culture. Conversely, deletion of the other identified TA modules had no effect on the level of persisters in stationary-phase culture, suggesting a probable redundancy (103). Redundancy was later confirmed when Maisonneuve et al. showed that single mutations of 10 independent TA modules had no effect on persister formation but a combination of mutations increased susceptibility toward ampicillin and ciprofloxacin (110). The same group used a flow cytometer to sort *E. coli* cells with low ribosomal activity that had been demonstrated to be enriched in persisters (93). The study of gene expression identified overexpression of known TA modules (*dinJ*, *yoeB*, and *yefM*) and also of *ygiU*, part of the *ygiUT* operon, which resembles a TA module. Overexpression of *ygiU* led to growth inhibition and also increased the levels of tolerance toward ofloxacin and cefotaxime but not tobramycin. Recently, a new type of TA module, type V, was associated with persistence in *E. coli*. In this case, the antitoxin GhoS masks GhoT toxicity through specific cleavage of *ghoT* mRNA, thereby preventing its synthesis (111). Interestingly, the authors also identified a possible interaction between GhoST and MqsR, a toxin that, upon inactivation, decreases formation of persister cells (112). They showed that the *ghoT*-encoded toxin transcript is resistant to MqsR, the toxin RNase encoded by *mqsRA*. Thus, when MqsR is induced, *ghoT* is still expressed and can contribute to persistence. Indeed, deletion of *ghoT* decreases MqsR-mediated persistence, and mild production of the GhoT toxin leads to persistence upon ampicillin treatment. Lastly, expression of the F-plasmid-based CcdAB TA system increases the persister level and could constitute a transmissible persistence factor (see below) (113).

Therefore, it appears that various TA modules have different and cumulative effects under different conditions, suggesting a certain level of redundancy. Another way to link TA modules and persister genesis would be through degradation of the unstable antitoxin, which ultimately would lead to activation of the toxin. In this regard, recent studies on the effects of the stringent reponse and Lon protease led to establishment of new connections between starvation and persistence.

(iii) Nutrient limitation and the stringent response. When a bacterial culture is kept in exponential phase with continuous dilution and constant medium renewal, persisters disappear (114). Conversely, at late stationary phase, the percentage of persisters increases and reaches a maximum, suggesting the importance of starvation in the genesis of persisters (114). This may be explained by indole production during stationary phase and nutrient limitation, leading to increased levels of *E. coli* persisters (115). Note that it was previously shown that indole production was increased in response to oxidative stress and antimicrobial exposure, through upregulation of the *tnaA* gene, which is responsible for indole synthesis (116).

Because the ppGpp-mediated stringent response is induced in cases of nutrient limitation, it was suspected of playing a role in the phenotypic switch of persisters 20 years ago. In 1995, ppGpp overexpression in *E. coli* was shown not only to increase antibiotic tolerance but also to inhibit peptidoglycan and phospholipid synthesis, thereby indicating a link between amino acid starvation, the stringent response, and antibiotic tolerance (117). Thus far, two major connections between the stringent response and persistence have been described: a defense against oxidative stress and an interaction with TA modules.

(a) The stringent response and oxidative stress defense. In *P. aeruginosa*, it was shown that *spoT* and *dksA* mutants had higher levels of ppGpp and were more tolerant toward fluoroquinolones (118). Furthermore, in *P. aeruginosa*, the stringent response is required for optimal catalase activity and mediates H_2O_2 tolerance during both planktonic and biofilm growth. Upon amino acid starvation, induction of the stringent response upregulates catalase activity (119). The demonstration of a link between the stringent response and oxidative stress defense is interesting, as ROS have been proposed to explain antibiotic-induced bacterial cell death (120–122). This subject remains a matter of intense debate and controversy, as other scientists recently published conflicting results that contradict this theory (123–126). However, it might be envisaged that because of the stringent response, bacterial persisters will be less damaged by ROS and thus exhibit tolerance (Fig. 4A). For instance, it was shown that in *P. aeruginosa* biofilms, a starvation-induced stringent response induces antioxidant mechanisms, such as superoxide dismutase (SOD) and catalase production, thus reducing ROS-induced damage and preventing cell death, ultimately leading to tolerance toward bactericidal antibiotics (89).

(b) The stringent response and TA modules. In the last 10 years, major studies have increased our understanding of the connection between the stringent reponse, TA modules, and persistence, and two main models have been proposed. For a comprehensive overview of this question, see reference 127.

The first model proposes that Lon protease plays a central role. The stringent response alarmone (p)ppGpp inhibits exopolyphosphatase, thus increasing the level of inorganic polyphosphate and ultimately inducing Lon protease activity (128, 129). It has been demonstrated that the Lon protease inactivates type II antitoxin molecules, including HipB (110, 130). Strikingly, all type II TA modules of *E. coli* encode mRNA endonucleases (mRNases) that degrade mRNA. The degradation of the related unstable antitoxin by Lon leads to an increased ratio of toxin to antitoxin, translation and replication arrest, and thus tolerance (Fig. 4A) (110). The same group demonstrated that (p)ppGpp stochastically triggers the activation of TA modules and thus controls the frequency of persisters (129).

Interestingly, a reverse model was proposed in 2003 for *E. coli*. It was suggested that free Hip toxin increases the level of ppGpp, thereby leading to altered gene expression and thus priming cells for the phenotypic switch (131). More recently, overexpression of HipA was shown to trigger growth arrest by inducing RelA-medi-

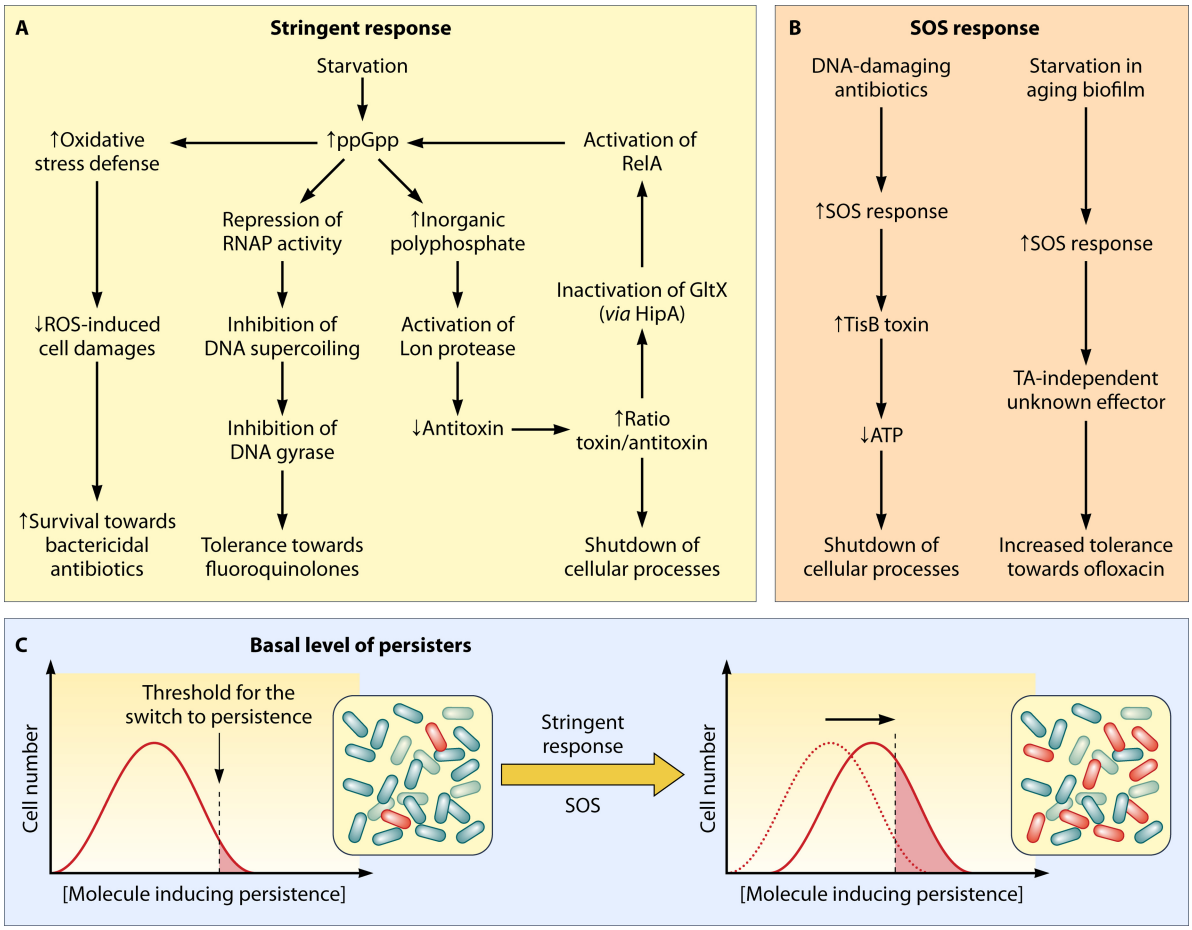

FIG 4 Main factors involved in generation of persisters. The stringent response (A) and the SOS response (B) are now considered pivotal in the generation of persisters. (C) Connection between stochasticity and persister genesis. In exponential-phase cultures, due to stochasticity, only a few bacteria reach the required threshold of a toxic molecule that is necessary to switch to the persister state (in red). Due to the factors described in panels A and B, there is an increased level of molecules inducing persistence; thus, more bacteria reach the threshold and become persisters. Note that most of these studies were conducted with planktonic bacteria. Panel C was inspired by a previous report (103).

ated synthesis of ppGpp (132). Suppression of ppGpp synthesis by use of chloramphenicol relieves Hip-mediated inhibition of DNA replication, thereby restoring vulnerability to β-lactam antibiotics (132).

These conflicting results were explained in a recent study in which the authors demonstrated that free HipA inactivates GltX (the glutamyl-tRNA synthetase) through phosphorylation. This event leads to the accumulation of uncharged $tRNA^{Glu}$ in the cell, which induces RelA-mediated activation of the stringent response (133). Ultimately, the level of ppGpp increases, leading to growth arrest and persister formation (Fig. 4A).

The second model to explain the connection between the stringent reponse, TA modules, and persistence suggests that the stringent response inhibits DNA supercoiling. Amato et al. studied the effects of carbon starvation on *E. coli* tolerance toward fluoroquinolones and demonstrated that, upon starvation, an increased level of cyclic AMP (cAMP) and/or a decrease in amino acid availability leads to an increase in ppGpp (100). Indeed, the interaction of cAMP and its receptor (cAMP receptor protein [CRP]) activates expression of *relA* and *dksA*. RelA and SpoT then synthesize ppGpp, which can repress the expression of stable RNA through an interaction with RNA polymerase (RNAP) and DksA, a small RNA polymerase binding protein (134, 135). This DksA-dependent repression of RNAP activity is associated with inhibition of DNA supercoiling, ultimately leading to inhibition of DNA gyrase and thus to tolerance toward fluoroquinolones (Fig. 4A) (100). Furthermore, the authors demonstrated that ppGpp-SpoT acted as a TA module on its own, with the following lines of argument: (i) the biochemical network of ppGpp suggests the possibility of bistability, and they confirmed this by using a kinetic model; (ii) ppGpp causes growth arrest through its interaction with RNAP; (iii) SpoT, the only known ppGpp hydrolase, cannot be knocked out in a $relA^+$ background; and (iv) ppGpp in excess of its antitoxin increases the level of persistence. In this ppGpp-SpoT model, persisters are cells with a higher level of ppGpp. ppGpp inhibits transcription, DNA replication, and DNA gyrase negative-supercoiling activity, thereby leading to fluoroquinolone tolerance. The same group recently demonstrated that a ppGpp-dependent pathway is also involved under biofilm conditions (136). Strikingly, they identified specificities regarding the importance of each involved protein or enzyme (136).

Finally, these results could lead to the design of antibacterial agents targeting the stringent response, such as RelA inhibitors, in order to increase persister cell mortality (137).

(iv) The SOS response. The SOS response, also called the DNA damage response, involves all the molecular mechanisms induced by chromosomal DNA damage caused by UV radiation or oxidative radicals. In 2004, a connection was established between the SOS system and tolerance. In that study, the authors demonstrated that inactivation of the *ftsI* gene product, penicillin-binding protein 3, by β-lactams induced SOS in *E. coli*, through the DpiBA two-component signal transduction system. This event, which requires the SOS-promoting *recA* and *lexA* genes as well as *dpiA*, transiently halts bacterial cell division, enabling survival upon otherwise lethal antibiotic exposure (138). A more recent study demonstrated that, in *E. coli*, ciprofloxacin at low concentrations triggered the SOS response system that leads to release of LexA-dependent repression of the *tisB* toxin gene (Fig. 4B). TisB can be inserted into the inner membrane and disrupt the proton motive force, which leads to a drop in the intracellular level of ATP. Subsequent shutdown of cellular processes is thought to be responsible for the observed higher level of persisters (139, 140).

Recently, it was shown that starvation and the SOS response can induce high biofilm-specific tolerance toward ofloxacin (141). In that study, a screen for *E. coli* mutants forming biofilms with increased tolerance toward antibiotics led to identification of amino acid auxotrophs displaying strong tolerance toward either ticarcillin or ofloxacin upon starvation. It was demonstrated that both functional RecA and cleavable LexA were essential for the starvation-induced biofilm-specific ofloxacin tolerance phenotype and that the SOS response was induced upon biofilm aging concomitantly with ofloxacin tolerance (Fig. 4B). Interestingly, a previous study from the same group showed that *recA* and other SOS response genes were significantly induced in mature biofilms compared to exponentially grown planktonic cells (142). Conversely, the ofloxacin tolerance of planktonic bacteria was likely due to a mechanism other than the SOS response, since a Δ*recA* mutant did not significantly impair the overall tolerance of either nonstarving or starving populations. The latter results strengthen the notion that induction of ofloxacin tolerance in starving biofilms is likely to involve mechanisms different from those currently described for planktonic cells (143, 144).

It is noteworthy that the SOS system is also induced by conjugative DNA transfer, an event that is enhanced in biofilms (145).

(v) Oxidative stress defense. Oxidative stress defense includes all bacterial mechanisms involved in protection against inadvertent by-products of aerobic metabolism, such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which are partially reduced oxygen species (146). ROS ultimately lead to DNA, protein, and lipid damages. Different defense mechanisms can be activated depending on the type of ROS. For example, activated macrophages produce O_2^- and H_2O_2, which induce bacterial SoxRS and OxyR regulons, respectively. This leads to activation of genes involved in ROS elimination and DNA repair (147). As discussed above, antibiotic-induced oxidative stress might play an important role in bacterial cell death. Therefore, it was deemed plausible that a way for persisters to survive in the presence of bactericidal antibiotics was to protect themselves from oxidative stress. For instance, flow cytometer analysis demonstrated that in a population of antibiotic-treated *E. coli* cells, persisters did not overproduce hydroxyl radicals, whereas most bacteria killed had a high level of hydroxyl radicals (148). Alongside the previously described stringent response-mediated defense against oxidative stress damages, another group reported that antioxidant strategies could lead to tolerance of bactericidal antibiotics. Indeed, H_2S has been demonstrated to increase the antioxidant capacity of Gram-positive and Gram-negative bacteria through suppression of the Fenton reaction and stimulation of SOD and catalase production (149).

Another group proposed a different scenario involving oxidative stress. They revealed that paraquat-induced oxidative stress led to an increase in the level of persisters surviving fluoroquinolone antibiotics, but not ampicillin or kanamycin (147). They showed that SoxRS induces overexpression of the AcrAB-TolC MDR pump, which can extrude fluoroquinolones (147). Thus, exposure to lower concentrations of fluoroquinolones may lead to persister formation. Furthermore, MDR pumps are also involved in protection against oxidative stress via the elimination of ROS (76).

Lastly, it was shown that oxidative stress was induced in biofilms independently of the presence of antibiotics (150). On the other hand, the SOS reponse is induced by ROS (151). Therefore, it can be envisaged that in biofilms, due to an increased level of oxidative stress, the SOS response is induced and increases the level of tolerance, as demonstrated in the case of ofloxacin (141).

(vi) Other cues leading to persistence. Aside from the above-described genetic mechanisms, different genes or regulators are involved in the switch to the persister state or, less precisely, in an increase in bacterial tolerance. In most of the following cases, the precise links between these genes and tolerance are not known.

(a) E. coli. In *E. coli*, the screening of a transposon mutagenesis library revealed that PhoU could play a major role in persistence (152). Inactivation of the *phoU* gene leads to decreased tolerance toward a wide range of antibiotics and various stresses, such as acidic pH, starvation, and heat. *phoU* expression is regulated by environmental changes, such as nutrient availability or the age of the culture, and its expression is decreased in rich media. *phoU* mutants exhibit upregulation of flagella, chemotaxis genes, and energy production enzymes, suggesting that the loss of the PhoU regulator renders the cells hyperactive. In case of starvation, *phoU* is expressed and affects genes involved in energy production and membrane transport. The precise effectors through which PhoU suppresses cellular metabolic activity are not known.

Another group used survival of ampicillin treatment as a screening method for an *E. coli* genomic mutant library and identified a hypertolerant clone with overexpression of the gene coding for the conserved aerobic *sn*-glycerol-3-phosphate dehydrogenase GlpD (153). Although deletion of *glpD* did not affect tolerance in exponential-phase cultures, it eliminated the majority of persisters in stationary phase. Two additional multidrug tolerance loci, *glpABC* and *plsB*, were identified through study of the pathway involving *sn*-glycerol-3-phosphate metabolism.

(b) P. aeruginosa. The importance of quorum sensing (QS) signals in tolerance was demonstrated in *P. aeruginosa* biofilms; indeed, their inhibition through mutations (Δ*lasR rhlR* QS receptor mutants) or use of inhibitors (furanones C-30 and C-56) led to decreases in tolerance toward tobramycin, H_2O_2, and phagocytosis by polymorphonuclear cells (PMNs) (154). The difference in tolerance might be related partly to a different biofilm structure, as QS plays an important part in biofilm architecture. However, similar observations were made in *P. aeruginosa* exponential-phase culture, as the level of persisters was significantly increased through the adjunction of exogenous phenazine pyocyanin or 3-OC_{12}-HSL (155). Pyocyanin, secreted by *P. aeruginosa* during stationary phase, reduced the growth of *P. aeruginosa* and exhib-

ited an effect on persister formation, during both the exponential and stationary phases, in a dose-dependent manner. Another structurally related compound (paraquat) had a similar effect, whereas phenazine-1-carboxylic acid (PCA) did not, despite strong structural similarity. Since a mutant unable to produce phenazine (Δ*phz1* and Δ*phz2*) had a similar level of persisters, it was suggested that redundant systems are present. The spectrum of action of each QS signal probably varies, as 3-OC_{12}-HSL increased the level of persisters only in strain PAO1, not in PA14 (155). In addition, small volatile QS compounds, such as 2′-aminoacetophenone (2-AA), have also been shown to influence persister cell accumulation (156).

In *P. aeruginosa*, in addition to QS signals, another locus has been identified. AmgRS is a two-component regulator, and its mutation was identified through screening of tobramycin-susceptible mutants. Indeed, *amgRS* mutations reduce planktonic and biofilm tolerance toward aminoglycosides (157). Transcription profiles suggest that AmgRS controls an adaptive response to membrane stress, possibly caused by aminoglycoside-induced insertion of misfolded proteins (157). The possible effectors of AmgRS-induced tolerance may be membrane proteases (HtpX and NlpD) and a protease-associated factor (YccA), which would help to eliminate misfolded proteins.

(vii) Stochastic gene expression. One mechanism that can be hypothesized for the persister switch is that of stochastic gene expression through fluctuations in transcription and translation rates despite stable environmental conditions (158). These variations result from two types of noise: (i) intrinsic noise, related to the nature of the process of gene expression and secondary to the rates of translation and mRNA and protein degradation; and (ii) extrinsic noise that varies from one cell to another and is caused by ribosome abundance or asymmetric distribution of proteins upon cell division. Indeed, even when all members of a planktonic culture are exposed to the same growth conditions, only a small fraction of them are persisters, suggesting the involvement of stochasticity (13). In this case, we speculate that at the population level, there exists a mean level of key persister regulatory protein expression associated with intracellular fluctuations due to the noises. For a small subset of bacteria, the level reaches a threshold, leading to the phenotypic switch (Fig. 4C). Then, when the population meets environmental triggers inducing stringent or SOS responses, the basal level of expression increases, leading to an increase in the percentage of cells reaching the threshold (94, 159). This hypothesis has been supported by experiments performed with TA modules that also demonstrate that the amount by which the threshold is exceeded determines the duration of dormancy (160).

(viii) Persister heterogeneity. As demonstrated above, many pathways, molecular mechanisms, and environmental factors are involved in the phenotypic switch that leads a bacterium to become a persister. Furthermore, some of these pathways are interconnected. Therefore, it is very likely that depending on the conditions prevailing during the switch, different types of persisters may appear, possibly simultaneously, in the same culture (100, 161). The type of antibiotic used to eradicate nonpersisters is a striking example of this and can influence gene expression, SOS induction, and the oxidative stress defense.

Even with homogeneous stresses similarly affecting the whole population, it was demonstrated that both a growth-arrest-mediated pathway and ppGpp-dependent pathways can be activated, leading to different types of persisters (100).

Ten years ago, the study of persisters by use of a microfluid device led to the hypothesis that two main types of persisters were produced: type I persisters were generated during stationary phase, with a prolonged lag phase before resuming growth upon transfer to rich media, whereas type II persisters were continuously produced independently of the growth phase (91). It is now clear that this view caught only a glimpse of the complexity and diversity of persisters.

Biofilms as a relevant environment for persister generation. Although most of the above-mentioned mechanisms were discovered under plankonic conditions, it is very likely that they are also involved in the generation of persisters in biofilms. For instance, due to nutrient limitations, the stringent response has already been shown to play a central role in *P. aeruginosa* biofilm recalcitrance (89). The SOS response is induced in biofilms and plays a role in biofilm recalcitrance toward antibiotics (141). On the other hand, due to the existence of biofilm-specific phenotypes and functions, caution should be taken in extrapolating persister data obtained under planktonic conditions to the biofilm lifestyle. Indeed, biofilm-specific mechanisms have been described and underline the complexity in the study of persisters (141).

The Biofilm Environment Favors Acquisition of Antibiotic Resistance

Patients suffering from biofilm-related infections are also exposed to nosocomial microorganisms present in their health care environment and selected by repeated antibiotic treatments. As a result, treatment of biofilm-related infections is difficult, not only due to biofilm recalcitrance toward antibiotics but also due to potential infection by multiresistent microorganisms carrying resistance genes, such as those encoding extended-spectrum β-lactamases (ESBLs) or methicillin resistance. In this case, biofilm formation and gene resistance issues can be additive as well as synergistic.

Biofilm formation favors horizontal gene transfer. Biological processes involved in horizontal gene transfer, such as conjugation, transformation, and transduction, have been demonstrated to be increased *in vitro* in biofilms (for a comprehensive review of this issue, see reference 162). Furthermore, while the presence of conjugative plasmids promotes biofilm formation, the biofilm lifestyle also increases plasmid stability and the range of mobile genetic elements (163). Hence, the presence of a biofilm is expected to facilitate the transfer of resistance genes, as demonstrated in an *in vitro* study, with an increased rate of transfer of a plasmid encoding CTX-M-15 (an ESBL) in a *K. pneumoniae* biofilm compared to the case under planktonic conditions (164). Transfer of a conjugative transposon (Tn*916*) carrying antibiotic resistance might also be responsible for acquisition of resistance mechanisms in biofilm bacteria (165). Transferability of genetic mobile elements between bacteria belonging to a multispecies biofilm has been described for a medical device implanted in a patient (166).

Interestingly, many transmissible DNA elements encode biofilm-promoting factors, including adhesins, such as conjugative pili, fimbriae, and autotransporter adhesins, and persistence factors, such as toxin-antitoxin modules. For instance, the F-plasmid-based CcdAB TA system increases the persister level and thus constitutes a transmissible persistence factor (113).

Impaired antibiotic diffusion through the matrix leads to bacterial exposure to subinhibitory concentrations of antibiotics. Due to biofilm architecture and drug diffusion issues, it is likely that some biofilm areas may be submitted at least transiently to subinhibitory concentrations of antibiotics. Exposure to subinhibitory concentrations of antibiotics is known to increase the likelihood of selecting resistant mutants (for a comprehensive review, see reference 167). Although it is generally assumed that selection of resistant bacteria occurs at antibiotic concentrations between the MIC of the susceptible wild-type population and that of the resistant bacteria, recent studies suggested that such selection could also occur at lower antibiotic concentrations (168). Furthermore, bacteria may produce hydroxyl radicals when exposed to sublethal concentrations of antibiotics (169). These hydroxyl radicals can induce the occurrence of mutations and help the organism to acquire resistance mechanisms. It has also been demonstrated that β-lactam antibiotics increase *E. coli* mutagenesis through RpoS-mediated reduction of replication fidelity (170). Similar findings have been made in *P. aeruginosa* during long-term experimental evolution, suggesting that CF patients who receive prolonged fluoroquinolone treatment might be exposed to this phenomenon (171).

Exposure to tobramycin at subinhibitory concentrations can increase the c-di-GMP level and biofilm formation, as demonstrated in *E. coli* and *P. aeruginosa*, through alterations in the level of c-di-GMP (172). Similar findings were made upon exposure of *Corynebacterium diphtheriae* to subinhibitory concentrations of erythromycin and, to a lesser extent, penicillin (173), but also for *P. aeruginosa* and imipenem or *S. aureus* and vancomycin or oxacillin (174, 175).

Recent studies also reported that antibiotics at subinhibitory concentrations can promote the transfer of mobile genetic elements, even though this has been demonstrated primarily under planktonic conditions. For instance, the fluoroquinolone-mediated SOS response may trigger expression, excision, and transfer of prophage genes (176). SOS induction may promote mobilization of various mobile elements, such as integrating conjugative elements (177). It has been shown that conjugation induces the SOS response and promotes antibiotic resistance through integron integration and activation *in vitro* (145, 178). More recently, an *in vivo* demonstration of this phenomenon was made through the identification of SOS-induced integrase expression ultimately leading to rearrangement of an integron gene cassette, full expression of a β-lactamase, and, thus, resistance toward ceftazidime (179).

Finally, as previously discussed, ciprofloxacin has been shown to increase the frequency of persisters through induction of SOS and, ultimately, production of the TisB toxin (139, 140). In general, preexposure to subinhibitory concentrations of antibiotics (0.2-fold MIC) increases the frequency of persisters with tolerance toward drugs belonging to different classes of antibiotics (ciprofloxacin, gentamicin, oxacillin, and vancomycin) (180). Although most of the data discussed here were generated with planktonic bacteria, it can be envisaged that this phenomenon is relevant in the case of reduced diffusion of antibiotics through the biofilm matrix.

Because biofilm persisters are more likely to survive antibiotic treatment, they are exposed to repeated rounds of different classes of antibiotics, inducing all the above-mentioned consequences and thereby amplifying the phenomenon (181). Although the interplay between biofilm recalcitrance, gene transfer, and spread of resistance could be of key importance in nosocomial settings, it remains to be demonstrated in clinical settings, or even in a relevant *in vivo* model of biofilm-related infections.

Genetic diversity within biofilms and its impact on biofilm recalcitrance. Various examples of genetic diversity occurring in biofilms have been described as influencing biofilm tolerance toward antimicrobial agents.

(i) Hypermutability. In *P. aeruginosa*, endogenous oxidative stress induces double-stranded DNA breaks in some cells within biofilms (150). Genetic variants arise when breaks are repaired by a mutagenic mechanism involving recombinatorial DNA repair genes. It was suggested that diversity and adaptability generated by this mechanism increase the ability of biofilm communities to adapt and survive in harsh environments; this mechanism is known as the "insurance effect" (150). Several genes, such as *katA* and *sodB*, also shown to be involved in protection against oxidative DNA damage, were downregulated under biofilm conditions (182). A similar mechanism has been described for the mucoid conversion of *P. aeruginosa* in CF patients. Indeed, free oxygen radicals, such as H_2O_2, released by PMNs can induce formation of mucoid variants through mutations in *mucA*, which encodes an anti-σ factor (183). This leads to deregulation of an alternative σ factor (σ22, AlgT, or AlgU) that is required for expression of the alginate biosynthetic operon (183). Hypermutators have been identified in clinical samples, and some of them are associated with specific mutations, such as *mutS*, belonging to the DNA mismatch repair (MMR) system (77). Aside from *mutL* and *uvrD*, which also belong to the MMR system, other genes were found to be mutated in hypermutators, such as *mutT*, *mutY*, and *mutM*, belonging to the DNA oxidative lesion repair system (184–186). Similar findings have been made in staphylococci, with mutability in biofilms that is 60-fold (*S. aureus*) and 4-fold (*Staphylococcus epidermidis*) higher than that under planktonic conditions (187). These mutations can lead to tolerance or resistance mechanisms.

(ii) Small-colony variants. SCV constitute a subset of the bacterial population that has been identified in a wide range of bacteria, including *S. aureus* and *P. aeruginosa* (29). They are associated with many diseases, including biofilm-related infections, such as osteomyelitis, chronic pulmonary infections in CF patients, and device-related infections. It has been demonstrated that their slow growth originates mainly from mutations associated with two types of defect: a deficiency in electron transport and a deficiency in thymidine biosynthesis. These SCV are frequently auxotrophic and are less susceptible to various antibiotics, depending on the metabolic alterations they exhibit (for comprehensive reviews of these issues, see references 188 and 29). As SCV may be present in biofilms, they may be involved in the global recalcitrance of the bacterial community. For instance, *P. aeruginosa* SCV have increased piliation, biofilm formation ability, and better adhesion to respiratory cell lines (189). Aside from SCV associated with mutations, phenotypic SCV have been described for *P. aeruginosa*. For instance, rough SCV (RSCV) of *P. aeruginosa* can be found *in vitro* and in clinical samples from CF patients and are associated with increased biofilm formation and antibiotic resistance (190). When RSCV are grown on antibiotic-free agar, wild-type revertants with a large colony size and a smooth appearance arise on the edges of the variant colonies. The regulatory protein PvrR of the two-component system PvrSR has been found to control conversion between antibiotic-resistant and antibiotic-

susceptible forms. Indeed, a PA14 Δ*pvrR* strain exhibited an increased frequency of resistant variants on kanamycin plates compared with the wild type. PvrR was later described as a phosphodiesterase modulating the c-di-GMP level in *P. aeruginosa*, suggesting the importance of c-di-GMP in controlling the onset of SCV (191).

ANTIBIOFILM STRATEGIES

Even prior to identification of the link between biofilms and human diseases, different therapeutic strategies were developed to prevent the occurrence of microbial colonization and to eradicate device-related infections, once established. However, most developments in the field of antimicrobial agents were based on planktonic studies, without taking into account the specificities of the bacterial biofilm lifestyle.

Currently Used Approaches Do Not Specifically Target Biofilm Bacteria

Hygiene, training, and reduction in the number of implanted devices. (i) Hygiene and training. Although hygiene is not a specific antibiofilm strategy, it prevents microbial contamination and thus adherence and subsequent biofilm formation. For almost all types of device-related infections, guidelines have been proposed to standardize procedures for device implantation and handling. For instance, the insertion of any central venous catheter (CVC) must be performed by trained personnel with maximum sterile barrier precautions, defined by the use of sterile gloves, cap, mask, sterile gown, and a sterile full-body drape (192, 193). The choice of skin disinfection solution and methods is also of key importance, and many reports suggest that alcohol-based antiseptics, such as alcohol-based chlorhexidine and alcohol-based povidone-iodine, are the most efficient solutions (192). Improvement of hygiene measures should always be attempted through definition and implementation of local clinical bundles for device insertion and handling, and in the case of CVC, dedicated infusion therapy teams have been developed for the education of patients and health care workers (192, 194, 195).

(ii) Early removal of an unnecessary device. Once a device is removed, the risk of bacterial contamination drops to zero. Therefore, at any time, physicians must discuss the benefits of maintaining an indwelling foreign body. For instance, a meta-analysis reported that use of an automatic reminder system for the removal of useless urinary catheters significantly decreased the incidence of catheter-associated urinary tract infections (CAUTI) (196). Of course, this approach is more difficult in the case of mandatory devices, such as pacemakers.

(iii) Systemic antibiotic prophylaxis during device insertion. Depending on the type of device, systemic antibiotic prophylaxis can be proposed in order to reduce the risk of microbial contamination. In that case, antibiotics are injected a few minutes before skin incision and are dedicated to eradicating any microorganisms that are not removed by skin disinfection. This approach is recommended in the case of surgically implanted devices, such as orthopedic and cardiac devices (197, 198).

Antibiotic coating of implanted devices. The principle of antibiotic coating of implanted devices is to deliver a locally high concentration of antimicrobials at the site of potential colonization (199). Depending on the type of device and the length of implantation, these antibiotic-coated materials can efficiently reduce the rate of colonization. The example of CVC can be taken to illustrate the benefits and limits of the antibiotic coating strategy. Indeed, for short-term CVC (<10 to 14 days of expected dwelling time), use of a coated CVC significantly reduces the risk of catheter-related infections and can be proposed when the infection incidence is still high despite implementation of all other preventive measures (192). Two types of coating have been developed: minocycline-rifampin and chlorhexidine-silver sulfadiazine. Comparative studies concluded that the former is more efficient than the latter (200–204). However, the benefits of antibiotic coating for long-term intravenous catheters (LTIVC) have not yet been demonstrated. Indeed, as these devices dwell for longer periods, the surfaces of LTIVC will be covered by a conditioning film composed of host cells or proteins that might limit the effect of any active surface. Furthermore, as soon as the antibiotic contained in the device is exhausted, antibiotic delivery stops. Antibiotic-coated surfaces have also been studied in animal and clinical studies of urinary catheters, endotracheal tubes, orthopedic devices, and vascular grafts, with contrasting clinical benefits (205–216). Thus, development of a coated surface that prevents bacterial colonization for a long time remains a challenge.

Mechanical removal of the source of infection. When clinicians are confronted with therapeutic difficulties or local and systemic complications, removal of the indwelling device may be required in the case of biofilm-related infection (194, 198, 212, 217). For short-term peripheral catheters, removal and replacement are easy, painless, and inexpensive. In contrast, removal of long-term catheters, pacemakers, or orthopedic prostheses is associated with complications for the patient, as well as with high costs. In the case of tissue-related infections, surgical removal of biofilm may be indicated for antibiotic failure. This is particularly the case for infective endocarditis (IE) and osteomyelitis, during which failure to cure the infection is an indication for surgery (218).

Optimization of the antibiotic regimen against biofilms. As physicians and clinical microbiologists became more aware of the importance of biofilms in infectious diseases, they attempted to define the antibiotics that were most active against biofilms and how these antibiotics should be prescribed so as to increase the likelihood of infection eradication.

One famous example of this challenging process is that of the rifampin-containing regimen, demonstrated to significantly improve the outcome of foreign-body-related *S. aureus* infections, first *in vivo* and then in clinical studies. Furthermore, fosfomycin and daptomycin are currently being investigated and might be promising candidates in the fight against foreign-body-related infections (219–221). In the case of prosthetic joint-related infection (PJI), *in vivo* models led to the demonstration that fluoroquinolones exhibited more penetration into the site of infection (222). Furthermore, *in vivo* models of foreign-body-related infections demonstrated that fluoroquinolones were the most efficient molecules when associated with rifampin (223). Based on these findings, fluoroquinolones have now become one of the mainstay treatments of PJI (223). A more recent example of an antibiotic associated with a potent antibiofilm effect is that of daptomycin. This bactericidal cyclic lipopeptide has an *in vitro* spectrum against Gram-positive pathogens through calcium-dependent disruption of membrane function, leading to potassium ion leakage and inhibition of DNA, RNA, and protein synthesis (224, 225). *In vitro* studies suggested that daptomycin may quickly penetrate *S. epidermidis* biofilms, that it is effective against biofilms,

and that it is bactericidal against stationary-phase and nondividing *S. aureus* (225–228). However, daptomycin alone was not able to cure the infection caused by methicillin-resistant *S. aureus* (MRSA) in tissue cage foreign body models, and its association with rifampin was not significantly better than a levofloxacin-rifampin association. Nevertheless, these 2 antibiotic combinations were more efficient than the previously recommended vancomycin-rifampin and linezolid-rifampin combinations (229). Using a similar methodology, another group demonstrated that daptomycin or rifampin as a single agent against MRSA was more effective than vancomycin or linezolid (221). Daptomycin has also been proposed for the treatment of catheter-related infections, and an *in vivo* study demonstrated that vancomycin and daptomycin were equally efficient at eradicating methicillin-resistant *S. epidermidis* (MRSE) catheter-related infections (230). Subsequently, a phase II clinical study was conducted using daptomycin antibiotic lock therapy (ALT) (see below), and it reported a cure rate of 85% (231). Comparative clinical studies are now expected to determine, for instance, whether daptomycin is more efficient and more rapid than vancomycin.

In the case of *P. aeruginosa* pneumonia in CF patients, optimized antibiotics may increase the likelihood of bacterial eradication, especially in early colonization. In that case, the early association of oral ciprofloxacin with inhaled colicin is associated with a reduced risk of chronic colonization (232, 233).

In addition to the choice of specific antibiotics, high dosages and prolonged treatment courses are required for biofilm-associated infections, as emphasized by cases of IE and osteomyelitis (218, 223).

Lock solutions to address the problem of biofilm recalcitrance. ALT is a strategy that relies on the injection of a highly concentrated (100× to 1,000× MIC) antibiotic solution into the lumen of a CVC. This solution should dwell for an extended time (at least 12 h) in order to eradicate any incoming bacteria. The chosen volume must allow coverage of the entire internal surface and therefore depends on the type of device, but it is usually small (between 2 and 5 ml). ALT can be used for prevention and treatment of catheter-related infections, but in most cases, its use is restricted to LTIVC. Indeed, microbial contamination of LTIVC occurs on the inner side of the device, defining intraluminal colonization. Thus, the highly concentrated antibiotic solution will be in close contact with the biofilm. On the other hand, in case of short-term CVC, contamination occurs mainly on the external surface of the device, defining extraluminal contamination. In that case, ALT cannot access the biofilm and is therefore useless.

(i) ALT for prevention of catheter-related bloodstream infections. As stated above, the ALT approach is restricted to prevention of LTIVC-related infections. A meta-analysis demonstrated that ALT composed of vancomycin reduced the risk of CRBSI (234). Other groups also assessed the combination of an antibiotic (minocycline) and a chelator, such as EDTA. Two studies in the pediatric oncology setting showed that minocyline-EDTA ALT was more effective than heparin for prevention of CRBSI (235, 236). Nevertheless, systematic use of ALT could lead to increased antibiotic resistance and should therefore be considered only for high-risk patients who have already experienced LTIVC-related infections (192, 237, 238). On the other hand, limited data are available concerning nonantibiotic lock solutions, such as ethanol and taurolidine, but they might also be used among high-risk patients (239, 240).

(ii) Conservative treatment of CRBSI with ALT. In cases of uncomplicated LTIVC-related BSI, a conservative treatment can be used based on ALT (90, 194). Indeed, if the clinical situation allows, catheter salvage is indicated in cases of reduced venous access or the potential presence of coagulation disorders (194). Such conservative treatment could avoid risks and reduce costs associated with a new surgical procedure. However, LTIVC removal is mandatory in cases of local or distant complications or in cases of infection caused by *S. aureus* or *Candida* spp., based on the high failure rate of treatment when the colonized catheter is retained (241). In other cases, conservative treatment using a combination of systemic antimicrobials and ALT can be considered (90, 194). Despite several limitations, there is a growing body of evidence favoring the use of ALT. For instance, a randomized, placebo-controlled study showed that ALT plus systemic antimicrobial therapy is more effective than systemic antimicrobial therapy alone for treating LTIVC-related BSI, although the result did not reach statistical significance due to the small sample size (242). In addition, large uncontrolled studies demonstrated high cure rates in patients with uncomplicated LTIVC-related BSI due to coagulase-negative staphylococci (CoNS) (89%) or Gram-negative rods (GNR) (95%) (241, 243, 244). Thus, the current indication for ALT is uncomplicated LTIVC-related BSI caused by CoNS or GNR (90, 194). Aside from commonly used antimicrobials in ALT, ethanol and daptomycin have recently been used for conservative treatment (see the previous section for daptomycin data). However, clinical data are still needed in order to recommend ethanol as a first-line compound for ALT (245–247).

Targeting Biofilm Recalcitrance: Progress and Perspectives

Currently used strategies have clearly improved the management of patients with indwelling devices in terms of both prevention and treatment of biofilm-related infections. However, many challenges remain before we can decrease the risk of microbial contamination on a surface or increase the likelihood of biofilm eradication. It is very likely that specific targeting of mechanisms known to play a role in biofilm recalcitrance will be a relevant strategy.

Preventive strategies. Within the limits of the different preventive approaches and the fact that most of them rely on the use of antibiotics, many efforts have been made to identify preventive strategies based on fundamental knowledge of mechanisms involved in bacterial adherence and biofilm formation (Fig. 5).

(i) Inhibiting microbial adhesion. Given the fact that without initial adhesion a biofilm cannot develop, the objective of inhibiting microbial adhesion is to impede the initial steps in biofilm formation.

(a) Material optimization, surface modifications, and biosurfactants. Inhibition of microbial adhesion to surfaces has been discussed extensively in several reviews (248–251). Here we simply describe the main approaches and develop a relevant example of each.

Since initial adhesion implies bacterial and surface factors, the physicochemical characteristics of the surface are of key importance in prevention of device-related infections. The physical nature of the material is important, as illustrated by human models of dental implant-associated biofilm (252). This experimental approach was used to demonstrate that bacterial adhesion to implant surfaces is significantly lower with a zirconium oxide surface than with pure titanium (Ti) (253). The biomaterial manufactur-

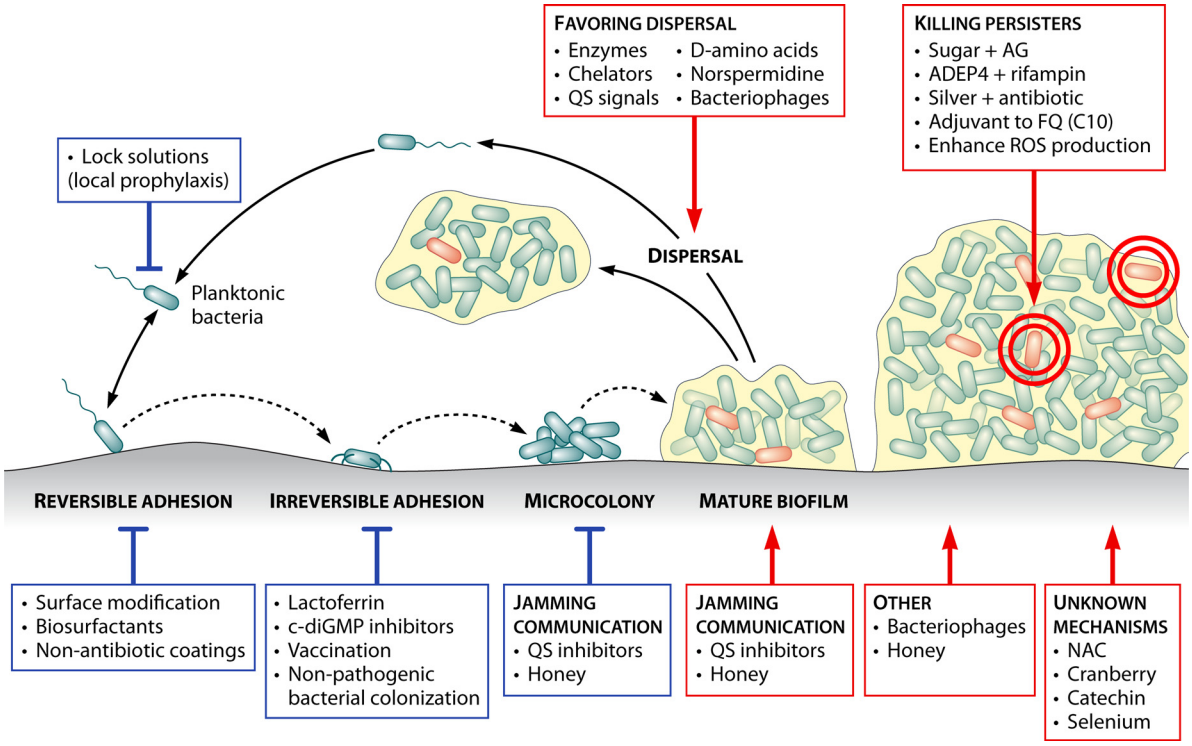

FIG 5 Antibiofilm strategies arising from fundamental research. Approaches to preventing formation of biofilms are depicted in blue; approaches to eradicating an established biofilm are shown in red. Persister cells are shown in red. AG, aminoglycosides; c-diGMP, cyclic di-GMP; FQ, fluoroquinolones; NAC, *N*-acetylcysteine; QS, quorum sensing; ROS, reactive oxygen species.

ing process can also modify roughness and physicochemical properties and thus affect bacterial adhesion. Indeed, electropolished stainless steel reduces bacterial adhesion compared to that with sandblasted steel (254). Furthermore, the choice of the polymeric material, even without any modification, is of key importance. Using a high-throughput microarray assay, bacterial adhesion was assessed on hundreds of polymeric materials and led to identification of materials comprising ester and cyclic hydrocarbon moieties (255). Coating of silicone with these materials significantly decreased *S. aureus* and *E. coli* adhesion *in vitro* and *in vivo* (255).

The physical architecture of the surface can also help to prevent microbial adhesion. For instance, the sharklet micropattern is a surface modification that mimics the microtopography of shark skin and has been shown to significantly reduce Gram-negative bacterial adhesion *in vitro* (256, 257).

Another major strategy for reducing bacterial adhesion is to modify the surface so it is protected by grafting antiadhesive molecules. One limitation to coated devices lies in the progressive coverage by a conditioning film made of proteins or cells from the patient. Thus, different attempts have been made to reduce not only microbial adhesion but also the deposition of host components or the occurrence of thrombosis. To do so, a peptide-based coating technology was proposed to modify the surface of Ti metal through noncovalent binding (258). In that study, a peptide (SHKHGGHKHGSSGK) possessing affinity for Ti was identified and coated with a pegylated analogue that efficiently blocked adsorption of fibronectin and *S. aureus* adhesion (258). Another group used lysozyme immobilized on polyethylene glycol monomethacrylate (PEGMA) to coat stainless steel surfaces and demonstrated that bacterial adhesion and albumin adsorption were reduced (259). Another surface modification using zwitterionic (a molecule with both positive and negative charges) nonleaching polymeric sulfobetaine (polySB) was associated with significant reductions in adherence and activation of platelets and white blood cells (260). This scaffold retains water on the surface of the catheter surface and reduces not only protein, host cell, and microbial adhesion but also thrombus formation *in vitro* and *in vivo* (260). Although these approaches have produced encouraging results, they still need to be evaluated in long-term settings.

Other surface modifications have been designed to kill bacteria once they stick to the surface, without using antibiotics. Two examples can be presented. First, poly(4-vinyl-*N*-alkylpyridinium bromide) covalently attached to glass slides and immobilizing polycationic chains (that have antibacterial properties) is able to kill airborne bacteria on contact (261). Second, single-walled carbon nanotube (SWNT) coatings were reported to have antimicrobial activities through cell membrane perturbation after an initial SWNT-bacterium interaction that ultimately leads to electronic structure-dependent bacterial oxidation and death (262).

Biosurfactants are surface-active molecules produced by many bacteria to inhibit adhesion of competitors. These compounds alter surface properties such as wettability and charge, thereby modifying bacterium-surface and/or bacterium-bacterium interactions (263, 264). Such molecules have therefore been studied as a possible surface modification in order to prevent bacterial adhesion. For instance, group 2 capsule and Ec300p, two hydrophilic high-molecular-weight polysaccharides produced by different *E. coli* strains, have been shown to prevent biofilm formation of

Gram-positive and/or Gram-negative pathogens (265, 266). Other molecules have been tested, including surfactin, rhamnolipids, and other molecules produced by lactobacilli and *Streptococcus thermophilus*, although these have not been identified clearly (see the reviews in references 263 and 267).

(b) Other types of nonantibiotic coatings. Because of limitations related to antibiotic coatings, such as their effect being restricted to nonresistant bacteria, different groups have tried to identify nonantibiotic coatings for preventing microbial adhesion. Use of antibody-releasing surfaces, such as a biomedical-grade polyurethane hydrogel coating containing solid dispersed bioactive antibodies, was proposed (268). The presence of antibodies reduced bacterial adhesion and enhanced bacterial killing during an *in vitro* opsonophagocytic assay using freshly isolated blood neutrophils (268). IgG opsonization was shown to inhibit bacterial adhesion by blocking cell surface attachment factors and altering the surface hydrophobicity of the bacterial cell (269). The main limitation of this approach was the short duration of antibody release (~24 h) (199), and an *in vivo* assessment of the preventive efficacy of this approach is still lacking. Since nitric oxide (NO) has antibacterial properties, NO-releasing surfaces have been proposed. An *in vivo* model using a medical-grade silicone elastomer with an NO-storing film implanted in rats led to a reduction in bacterial colonization after *S. aureus* challenge (see below for the effects of NO on dispersal) (270). Other vascular catheter coatings have been studied, such as the association of triclosan (an antiseptic) and dispersin B (an antibiofilm enzyme) (see below) to prevent *S. aureus* colonization *in vitro* and *in vivo* (271). Triclosan-loaded urinary catheters have also been studied successfully *in vivo* for the prevention of *Proteus mirabilis* CAUTI (272). Another antiseptic-coated catheter containing gendine demonstrated significant reductions in *E. coli* adhesion both *in vitro* and *in vivo* in a CAUTI model (273).

(c) Inhibition of production of adhesins. Different molecules have been designed to specifically inhibit the production of bacterial adhesins involved in biofilm formation. As an example, ring-fused 2-pyridones inhibit curli biogenesis in uropathogenic *E. coli* (UPEC) and prevent polymerization of the major curli subunit protein, CsgA. Some of them also have a pilicide effect, i.e., inhibition of the assembly of type 1 pili, which is required for pathogenesis during urinary tract infection via the FimH adhesin exposed at the tips of the pili. One molecule, FN075, has been demonstrated to block biogenesis of both curli and type 1 pilus, to inhibit biofilm formation, and to attenuate virulence in a mouse model of urinary tract infection (274).

(d) Blocking of interaction of adhesins with their receptors. Another approach is to specifically target the FimH type 1 pilus lectin of UPEC, which mediates bacterial colonization, invasion, and formation of recalcitrant intracellular bacterial communities in the bladder epithelium (275). Low-molecular-weight mannose-derived compounds called mannosides were designed and adapted for oral administration. Indeed, the mannose binding pocket of FimH is composed of amino acids that are invariant in all strains of *E. coli*. When tested in a mouse model, the mannosides were able to prevent UTI when given prophylactically or to treat an established chronic urinary tract infection (275). These molecules have been demonstrated to prevent and treat UPEC CAUTI in a mouse model (276). Furthermore, synergistic action was noted between mannosides and trimethoprim-sulfamethoxazole, suggesting the utility of adjuvant approaches in this setting (276). Prophylactic administration of a mannoside molecule, compound ZFH-04269, was recently demonstrated to significantly reduce bacterial colonization of the bladder and to prevent acute UTI caused by an epidemic multidrug-resistant UPEC ST131 clone. Treatment of chronically infected mice with the same FimH inhibitor lowered their bladder bacterial burdens over 1,000-fold (277).

Aside from direct administration of an adhesin inhibitor, other authors proposed covering a surface with an adhesin inhibitor. For instance, coverage of a surface with multivalent galabiose derivatives significantly inhibits adhesion of *E. coli* through inhibition of P fimbriae *in vitro* (278). The main limitation of this approach is the multiplicity of structures involved in bacterial adhesion. However, one way to circumvent this issue is to use multivalent inhibitors linked to a scaffold of glycopolymers, glyconanoparticles that may permit inhibition of several adhesins at the same time (279–281).

(e) Use of lactoferrin. Lactoferrin is a component of innate immunity found in numerous body fluids (tears, milk, and respiratory secretions) and is an iron chelator that has been demonstrated to inhibit irreversible adhesion of *P. aeruginosa in vitro* (282). Through iron chelation, lactoferrin stimulates twitching motility, during which bacteria wander across the surface instead of forming microcolonies and biofilms. Indeed, iron metabolism and transport are required for normal biofilm development (283). Interestingly, *S. aureus* biofilm production is induced under iron-restricted conditions and is repressed by iron via a Fur-independent mechanism, suggesting that the effect of lactoferrin may depend on the bacterial species (284). The effect of lactoferrin can be increased by the adjunction of xylitol, a rare sugar that inhibits the ability of the bacteria to produce siderophores under conditions of iron restriction (285). Such an association could be proposed in case of chronic wounds colonized by *P. aeruginosa* biofilm (286). Assessment of the antibiofilm efficacy of other known iron chelators and development of new iron chelators targeting biofilms might be future antibiofilm strategies to consider.

(f) Inhibition of c-di-GMP biosynthesis. The inhibition of c-di-GMP biosynthesis by diguanylate cyclase (DGC) is also promising, in light of its importance in the shift from the planktonic to the biofilm lifestyle. Indeed, blocking c-di-GMP biosynthesis may keep bacteria in the planktonic state. Screening for DGC inhibitors identified sulfathiazole (287). Sulfathiazole inhibits formation of biofilms *in vitro* and indirectly inhibits DGC through inhibition of tetrahydrofolate biosynthesis, which affects the pool of thymidine, and DNA synthesis, rather than via enzymatic inhibition (287, 288). More direct inhibition of DGC was identified in *V. cholerae*, *P. aeruginosa*, and *Acinetobacter baumanii* through high-throughput screening (289, 290). Several molecules inhibiting DGC and biofilm formation of these three pathogens were identified; however, the tolerance and toxicity of most of these compounds remain to be assessed.

(g) Physical approaches. Physical approaches have been developed to prevent biofilm formation on catheters, including low-energy surface acoustic waves and iontophoresis as preventive measures (291, 292). In the latter case, urethral catheters are modified in order to deliver a current to electrodes located on the catheter tip, leading to production of ions of soluble salts and allowing formation of a local biocide. After 3 weeks, this approach significantly reduced the bacterial burden in urine. Surface acous-

tic waves have also been proposed for the eradication of biofilms, in conjunction with antibiotics (293).

(ii) Jamming communication through inhibition of quorum sensing. The objective of the jamming approach is to inhibit biofilm formation by altering the progression from initial attachment to microcolonies and development of a mature biofilm. As quorum sensing (QS) is a key component of biofilm communication, many authors have speculated that interfering with QS signals might alter biofilm maturation, thereby leading to easier eradication. However, the main limitation of QS inhibition is the spectrum of action, which depends on the type of QS system used by the microorganism responsible for the infection.

RNAIII-inhibiting peptide (RIP), a compound interfering with *S. aureus* QS, efficiently prevents CVC-related infection *in vivo*, alone or in association with antibiotics, in a rat model (294). Similar *in vitro* and *in vivo* data have been published for *S. epidermidis* (295). Aside from the CVC situation, RIP has been assessed in other types of biofilm-related infections, such as biofilms formed by *S. epidermidis* or *S. aureus* on chronic wounds, where it reduces the healing time *in vivo* (296).

In *P. aeruginosa*, different molecules have been developed to interfere with QS signals. Azithromycin has poor antimicrobial activity against *P. aeruginosa*, but it interferes with *lasI*-mediated QS signals (297–299). It was shown to inhibit *P. aeruginosa* biofilm formation and virulence factor expression both *in vitro* and *in vivo* (297, 300). Clinical studies in CF patients colonized by *P. aeruginosa* demonstrated an improvement in respiratory function and a reduced number of exacerbations compared with the placebo (301). Nevertheless, recent data suggest that the chronic use of azithromycin might be associated with side effects, such as ototoxicity and an increased level of bacterial resistance (302, 303). Possible cardiovascular toxicity has been described, with conflicting results (304). As acyl-homoserine lactones (AHLs) play a key role in the development of *P. aeruginosa* biofilms, inhibitors of these autoinducers have been developed (305). An *N*-acyl-homoserine lactone hydrolase (BpiB05) was identified through screening of a soil metagenome, and it inhibits *P. aeruginosa* biofilm formation *in vitro* (306). Along with AHLs, synthetic furanones derived from an algal metabolite now constitute potential prevention candidates, as they inhibit Gram-negative bacterial QS through their fixation to LasR and inhibition of the action of AHLs (288, 307). *In vitro* and *in vivo* studies reported reduced biofilm formation, virulence factor expression, and antibiotic tolerance of *P. aeruginosa* biofilms exposed to furanones (308, 309). However, furanones have a narrow spectrum of activity, as they are efficient only against bacteria that share this QS signaling pathway (309, 310). Furthermore, the use of halogenated furanones remains hampered by their carcinogenic effects as well as poor stability in aqueous solutions.

Through screening of chemical libraries, different QS inhibitors have been identified, such as garlic extract and 1-isothiocyanato-3-(methylsulfinyl)propane, also known as iberin, from horseradish (311, 312). The compound isolated from garlic, ajoene (4,5,9-trithiadodeca-1,6,11-triene-9-oxide), was shown to increase *P. aeruginosa* biofilm susceptibility to tobramycin and PMN activity *in vitro* (311, 313). *In vivo*, mice treated with garlic extract for 7 days, with the initial 2 days being given before *P. aeruginosa* instillation, had more severe initial inflammation but better bacterial clearance of the infection than placebo-treated mice (313). Another example of a QS inhibitor identified in vegetal matter is green tea epigallocatechin gallate, which was shown to reduce QS, biofilm development, and virulence factor production of *P. aeruginosa* through inhibition of the enoyl-acyl carrier protein reductase (ENR), ultimately leading to a reduction in $3OC_{12}$-HSL of the *las* QS system (314).

Lastly, different authors have proposed grafting enzymes able to digest QS signals, called quorum-quenching molecules, on the surface in order to inhibit bacterial adhesion (315, 316).

(iii) Vaccination. The goal of vaccination is to induce the production of antibodies against bacterial biofilm antigens, such as structures involved in adhesion or biofilm maturation. This strategy requires predefining groups of patients about to be exposed to the risk of biofilm-associated infection and treating them before exposure. A relevant example is the scheduled implantation of devices such as heart prosthetic valves, pacemakers, and prosthetic joints. This strategy may also be relevant for patients exposed to chronic tissue-associated infections, such as CF patients or patients suffering from recurrent UTI. Ideally, biofilm-specific antigens should be used to increase the effect of vaccination. Choosing the right antigen remains an arduous task due to the obvious redundancy of bacterial appendages involved in adhesion and biofilm formation. Therefore, current strategies are aimed at using an antigenic cocktail (317, 318). For UTI, *in vitro* and *in vivo* studies demonstrated that immunization with FimH or components of the P pilus from UPEC reduced *in vivo* colonization of the bladder mucosa (319, 320). For CVC-related infections, a rat model enabled assessment of immunization of rats prior to catheter insertion, leading to a protective effect in bacterial colonization of the device by *S. aureus* or *S. epidermidis* (321). In that study, two different antigens were used: SERP0630 (MenD) (for *S. epidermidis*) and SACOL1138, or iron-regulated surface determinant B (IsdB) (for *S. aureus*). With *S. aureus*, a recent study reported that extracellular proteins found in the biofilm matrix could induce a protective immune response that prevented subsequent infections (322).

Aside from vaccination aimed at preventing bacterial adhesion, it has also been suggested that vaccination will increase the likelihood of biofilm eradication (323). Antigens were chosen (glucosaminidase, an ABC transporter lipoprotein, a conserved hypothetical protein, and a conserved lipoprotein) because they are upregulated in biofilms both *in vitro* and *in vivo*. In a rabbit osteomyelitis model, the association of antibiotics and vaccination significantly increased the rate of therapeutic success (323). In that model, vaccination was initiated 30 days prior to the onset of infection, thus reducing the impact of the findings.

(iv) Use of nonpathogenic bacteria to prevent colonization. The use of nonpathogenic bacteria to prevent colonization relies on nonpathogenic bacteria that are able to efficiently colonize a surface and thus compete with other bacterial pathogens and prevent their adhesion (324). The best-documented case is the *E. coli* 83972 strain, which is responsible for asymptomatic bacteriuria (ABU). This strain lacks most virulence factors and UTI-associated adhesins and fails to induce bladder inflammation (325). It was observed that antibiotic treatment of patients with ABU led to a paradoxical increase in the risk of UTI by other bacteria, thus leading to the hypothesis that *E. coli* 83972 bladder colonization could prevent the occurrence of UTI. Since then, different clinical studies have demonstrated that bladder inoculation with *E. coli* 83972 has beneficial effects. For instance, in a clinical pilot study, patients with incomplete bladder emptying and recurrent UTI

were randomized to receive blinded bladder inoculations with *E. coli* 83972 or saline (326). Inoculated patients experienced significantly fewer UTI during the 12 months following inoculation.

Hence, several promising strategies have been developed to prevent microbial adhesion and biofilm formation. Only a few of them have undergone *in vivo* efficacy tests, and for most of them, the precise mechanisms of action remain unknown (Table 2).

New approaches to eradicating already formed biofilms. Most currently used strategies for biofilm eradication were developed even before the identification of the importance of biofilms in human medicine. Many clinical studies have been conducted using robust endpoints, such as clinical and/or microbiological cures and infection recurrence. Therefore, major improvements have already been made in these fields. However, several therapeutic failures are still being observed, even when patients are managed at reference centers. First, cure rates never reach 100%, and the risk of treatment failure can even reach 50%, depending on host and pathogen factors. Furthermore, most currently used strategies rely on antibiotics, thereby increasing the selective pressure and the risk of antibiotic resistance. Finally, prolonged treatment is frequently required, leading to considerable medical cost and toxicity.

Nonantibiotic compounds, used alone or in combination with antibiotics to increase the likelihood of biofilm eradication or to reduce the length of treatment, are therefore viewed as modern "holy grails." Recent breakthroughs in understanding biofilm recalcitrance have given rise to plausible therapeutic strategies.

(i) Induction of dispersal. Inducing dispersal is a tempting strategy; indeed, biofilm bacteria lose some of their antibiotic tolerance when they return to a planktonic state and are exposed to the host immune system (14). However, the dispersal approach needs to be associated with the use of systemic antibiotics, as release of biofilm bacteria into the bloodstream can lead to severe sepsis (327, 328). Several strategies have been proposed to induce biofilm dispersal.

(a) Enzymes for dissociating components of the ECM. Because ECM plays an important role in maintaining biofilm stability and structure, it has been speculated that use of an enzyme able to dissociate or digest ECM components would lead to dispersal of the biofilm. Two main targets have been identified: poly-*N*-acetylglucosamine (PNAG) and extracellular DNA. Dispersin B is a hexosaminidase produced by *Aggregatibacter actinomycetemcomitans* that hydrolyzes PNAG, an important component of *S. epidermidis* ECM. It is therefore effective against biofilms formed by this bacterial species (329, 330). It should be noted that PNAG is also produced by some *S. aureus* strains, as well as *E. coli*. On the other hand, given the important role played by extracellular DNA in the structure of the biofilm matrix (331, 332), DNase I, an enzyme that degrades DNA, was efficiently used to dissolve biofilms from a broad range of bacteria, including *P. aeruginosa*, *S. aureus*, and *Gardnerella vaginalis* (330, 333, 334).

However, the enzyme-based approach is associated with two limitations: (i) the restricted spectrum of action and (ii) the risk of immunization against these molecules.

(b) Divalent cation chelators. Since divalent cations play a key role in maintaining biofilm ECM stability and cohesiveness, another approach is to use chelators such as EDTA and citrate (335, 336). For instance, calcium ions cross-link alginate, and the calcium concentration was shown to be critical for maintaining *P. aeruginosa* biofilm resistance toward compressive stresses (337). Iron has also been demonstrated to be an important cross-linker of the ECM (338). However, little is known about the mode of action and precise effects of chelators on biofilms. One study in 1983 reported that the addition of EGTA, a specific calcium chelator, led to immediate detachment of a mixed bacterial film from the walls of a recycle tube reactor (339). EDTA at 50 mM has been shown to induce dispersal and lysis of *P. aeruginosa* biofilm bacteria (340). Strikingly, addition of calcium, iron, or magnesium inhibited the phenotype. In that study, EGTA led to the same dispersal phenotype, but without inducing lysis. Furthermore, citrate and EDTA were also shown to exhibit direct bactericidal effects against planktonic bacteria (340, 341).

Many *in vitro* studies have reported a synergistic antibiofilm effect of EDTA combined with gentamicin or minocycline-25% ethanol (340, 342, 343). Using a rat model of biofilm-related infection with a totally implantable venous access port (TIVAP), it was recently shown that the gentamicin-EDTA combination was the most effective lock solution compared to gentamicin alone, EDTA alone, or ethanol (70%) (87, 344). Gentamicin-EDTA is therefore a potential lock solution able to cure highly tolerant biofilms and eradicate persistent bacteria, thereby preventing recurrence of Gram-positive as well as Gram-negative bacterial biofilms on TIVAP (344).

(c) QS signals. While QS signaling can be targeted to interfere with biofilm formation, some QS signals can also be used to trigger dispersal of a biofilm. In *S. aureus*, the *agr* (accessory gene regulator) QS system is strongly expressed by the bacterial population at the moment of dispersion. Artificial stimulation of this system, through adjunction of autoinducing peptide (AIP), leads to *S. aureus* biofilm dispersal (345). *In vivo* murine models also helped to reveal the effect of RIP (a quorum sensing inhibitor) in combination with teicoplanin against methicillin-resistant *S. aureus* in the setting of chronic wound biofilm colonization (346). Lastly, with *P. aeruginosa*, analysis of spent medium led to the discovery of a short-chain fatty acid that is implicated in bacterium-bacterium communication (*cis*-2-decenoic acid) and is able to induce dispersal in a wide range of Gram-positive as well as Gram-negative bacteria (347).

Like preventative QS signal jamming, this strategy is limited by the spectrum of action of each QS molecule.

(d) Other strategies for inducing dispersal. Bacillus subtilis produces a mixture of D-amino acids (D-leucine, D-methionine, D-tyrosine, and D-tryptophan) that disperse existing biofilms through release of TasA, an amyloid fiber that links biofilm bacteria together. D-Amino acids also prevent biofilm formation by *S. aureus* and *P. aeruginosa* (348). While the same group reported that, in fact, the effect of D-amino acids on *B. subtilis* biofilm dispersal is due to their misincorporation into proteins via a mutation in the *dtd* gene, encoding D-Tyr-tRNA deacylase (349), the mechanisms by which D-amino acids affect biofilm formation by *S. aureus* and *P. aeruginosa* remain to be elucidated. The same group reported that *B. subtilis* also produces an additional biofilm disassembly factor, norspermidine, that interacts directly and disrupts exopolysaccharides. Strikingly, norspermidine also prevents biofilm formation by *B. subtilis*, *E. coli*, and *S. aureus* (350). However, another group recently published conflicting results demonstrating that norspermidine is not involved in biofilm disassembly (351).

Nitric oxide (NO) can induce dispersal of *P. aeruginosa* biofilms through induction of phosphodiesterases and, ultimately, a reduction in c-di-GMP levels (352, 353). Exposure to the NO

TABLE 2 Antibiofilm strategies originating from fundamental research

Mode of action	Comments[a]		Reference(s)
	In vitro	*In vivo*	
Inhibition of microbial adhesion			
Material optimization	Physical nature of the substrate (zirconium versus titanium) and its handling (electropolished versus sandblasted) reduce bacterial adhesion	Physical nature of the substrate (zirconium versus titanium) and its handling (electropolished versus sandblasted) reduce bacterial adhesion	254, 252, 253, 375
Specific polymeric material	Reduction of *S. aureus* and *E. coli* adhesion	Reduction of *S. aureus* and *E. coli* adhesion	255
Modify physical architecture of the surface (sharklet micropattern)	Reduction of *E. coli*, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae* adhesion		256, 257
Grafting of antiadhesive molecules	Reduction of *S. aureus* adhesion and fibronectin adsorption on titanium grafted with specific peptides	Nonleaching polymeric sulfobetaine reduces bacterial adhesion and thrombus formation on the surface of a catheter	258, 260, 259
Killing of bacteria upon contact	Polycationic chains attached to glass slides or single-walled carbon nanotubes		261, 262
Biosurfactants	Reduction of biofilm formation (*E. coli*, *P. mirabilis*, *Candida* spp., *S. aureus*)		265, 267, 266
Other nonantibiotic coatings			
Antibody-releasing surfaces	IgG opsonization inhibits bacterial adhesion and increases bacterial killing by neutrophils		376, 268
Nitric oxide-releasing surfaces		NO-coated surfaces in rats show reductions in *S. aureus* colonization	270
Association of antiseptic (triclosan) and dispersin B	Prevention of *S. aureus* colonization	Prevention of *S. aureus* colonization on vascular catheters	271
Gendine-coated surfaces	Reduction of *E. coli* adhesion	Reduction of *E. coli* adhesion on a urinary catheter model	273
Inhibition of production of adhesins (FN075, ring-fused 2-pyridones)	Inhibits curli biogenesis in uropathogenic *E. coli* and inhibits biofilm formation	Inhibits biofilm formation and attenuates virulence in a mouse model of urinary tract infection	274
Blocking the receptor of adhesins			
Mannosides	Target the FimH type 1 pilus lectin of UPEC	Prevent UTI or treat an established chronic urinary tract infection	275, 276, 277
Coverage of a surface with multivalent galabiose derivatives	Inhibits adhesion of *E. coli* through inhibition of P fimbriae		280, 278
Lactoferrin	Inhibits irreversible adhesion of *P. aeruginosa* (via iron chelation)		282
EDTA (divalent cation chelator)	Prevents biofilm formation	When associated with minocycline, reduces the risk of catheter-related infections	377
Inhibition of c-di-GMP biosynthesis			
DGC inhibitor (sulfathiazole)	Indirect effect through inhibition of tetrahydrofolate biosynthesis		287, 288
Direct inhibitors of DGC	Inhibits DGC and biofilm formation of *V. cholerae*, *P. aeruginosa*, and *A. baumannii*		289, 290
Physical approaches			
Low-energy surface acoustic waves		Reduction of *E. coli* biofilm formation on urinary catheters	292
Iontophoresis		Reduction of *E. coli* biofilm formation on urinary catheters	291

Jamming communications			
Quorum-sensing inhibitors			
RNAIII-inhibiting peptide (RIP)	Reduces adhesion and virulence of *S. aureus*	With *S. aureus* and *S. epidermidis*, reduces colonization of CVC (rats) and improves healing of chronic wounds (mice)	294, 296
Azithromycin	Inhibits biofilm formation and virulence of *P. aeruginosa*	Inhibits biofilm formation and virulence of *P. aeruginosa*	297, 301
Acyl-homoserine lactone inhibitors or hydrolases	Reduce *P. aeruginosa* biofilm formation		306, 305
Furanones	Inhibit biofilm formation and virulence of *P. aeruginosa*	Reduce *P. aeruginosa* virulence	308, 288
Garlic extract (ajoene)	Increases *P. aeruginosa* biofilm susceptibility to tobramycin and PMN activity	Increases bacterial clearance in a mouse *P. aeruginosa* infection model	154, 311
Green tea epigallocatechin gallate	Reduction of QS, biofilm development, and virulence factor production of *P. aeruginosa*		314
Quorum-quenching molecules	Inhibit bacterial adhesion		315, 316
Vaccination			
Prevent initial adhesion		Prevents CVC colonization by *S. epidermidis* and *S. aureus* in rats	321
Improve eradication of a biofilm-related infection		In a rabbit osteomyelitis model, the combination of antibiotic and vaccination significantly increased the rate of therapeutic success	323
Other preventive measures			
Using nonpathogenic bacteria (*E. coli* 83972)		Inoculated patients experienced fewer UTI during the 12 months following inoculation	325, 326
Cerium nitrate, chitosan, and hamamelitannin	Prevent formation of biofilms of *S. epidermidis*, *S. aureus*, *Acinetobacter baumannii*, and *Candida albicans*	Prevention of formation of biofilms of *S. epidermidis*, *S. aureus*, *Acinetobacter baumannii*, and *C. albicans* in a subcutaneous CVC model	378, 379
Allicin from garlic	Inhibits PIA biosynthesis and biofilm development of *S. epidermidis*		380
Sulfhydril compounds (dithiothreitol or cysteine)	Inhibit *S. aureus* biofilm formation through inhibition of *ica*		381
Ginseng extract	Prevents and disperses *P. aeruginosa* biofilm; stimulates swimming and twitching motilities	Oral administration of ginseng enhances phagocytosis of *P. aeruginosa*	382
Blockade of DNA replication (5-fluorouracil [5-FU])	Reduces the virulence and biofilm formation of *P. aeruginosa* and *E. coli*	5-FU-coated catheters in clinics for prevention of catheter colonization	383, 384, 385
Favoring dispersal			
Enzymes			
DNase I	Favors dispersion of *S. aureus*, *P. aeruginosa*, or *Gardnerella vaginalis*	Inhibits *Gardnerella vaginalis* biofilm formation	333, 330, 334
Dispersin B (against PNAG)	Favors dispersal of *S. epidermidis* more than that of *S. aureus*	With triclosan (antiseptic), reduces *S. aureus* colonization in a rabbit CVC model	329, 330
Divalent cation chelator (EDTA)	Dispersal and lysis of *P. aeruginosa* biofilm bacteria	Gentamicin-EDTA combination eradicates Gram-positive and Gram-negative biofilms	340, 87
Modulation of quorum sensing			
Autoinducing peptide	Induces *S. aureus* biofilm dispersal		345
RIP		RIP plus teicoplanin was used against methicillin-resistant *S. aureus* biofilms in chronic wounds	346
cis-2-Decenoic acid	Induces dispersal of *S. aureus*, *E. coli*, *C. albicans*, and *Streptococcus pyogenes* biofilms		347

(Continued on following page)

TABLE 2 (Continued)

Mode of action	Comments[a]		Reference(s)
	In vitro	*In vivo*	
D-Amino acids	Induce *B. subtilis* biofilm dispersal and prevent biofilm formation by *S. aureus* and *P. aeruginosa*		348
Norspermidine	Induces *B. subtilis* biofilm dispersal and prevents biofilm formation by *S. aureus* and *E. coli*; ongoing controversy		351, 350
Nitric oxide	Induces dispersal of *P. aeruginosa* biofilm		352, 353
Phages	Phage PT-6 produces an alginase that favors *P. aeruginosa* dispersal		362
Antipersister compounds			
Sugars (mannitol or fructose) plus aminoglycosides	Increase mortality among persisters (*S. aureus*, *E. coli*, and *P. aeruginosa*)	Increase aminoglycoside efficacy against *E. coli* biofilms (catheter-associated urinary tract infection)	86, 355
Silver plus antibiotics	Increases the effect of gentamicin, ofloxacin, or ampicillin against planktonic and biofilm persisters	Increases the effect of gentamicin, ofloxacin, or ampicillin against planktonic and biofilm persisters	356
ADEP4 plus rifampin	Eradicates *S. aureus* biofilms	Eradicates *S. aureus* biofilms	88
C10 plus norfloxacin	Increases mortality among *E. coli* persisters		386
Farnesol	Reduces *S. aureus* tolerance toward gentamicin		387
Bacteriophages	Phage T4 against *E. coli* or phage F116 against *P. aeruginosa*	Reduce mouse ileum colonization by *E. coli*	358, 359, 388, 364, 361
Other compounds for eradicating biofilms			
N-Acetylcysteine	Eradication of biofilms formed by Gram-positive and Gram-negative pathogens	Associated with tigecycline in a clinical study	389, 390, 391
Honey	Antibiofilm (*Enterobacter cloacae*, *P. mirabilis*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes*) and anti-inflammatory effects; inhibits QS signals and represses curli genes (*csgBAC*) or indole biosynthesis	Adjunct therapy for chronic wound care	392, 393, 394, 395, 396, 397, 398, 399
Cranberry or selenium, with or without ciprofloxacin		Treatment of chronic bacterial prostatitis	400, 401
Catechin (extract from Chinese tea) plus ciprofloxacin		Treatment of chronic bacterial prostatitis	402
Synthetic antimicrobial peptidomimetics (SAMP)	Active against staphylococcal biofilms		403
Electrical current (in the presence of NaCl)	Active against *S. epidermidis* or *P. aeruginosa* biofilms, through generation of free chlorine and, ultimately, hypochlorous acid and hypochlorite		404
Ultrasound-mediated microbubbles	Enhance vancomycin effect against *S. epidermidis* biofilms		405

[a] CAUTI, catheter-associated urinary tract infection; CVC, central venous catheter; NO, nitric oxide; PNAG, poly-*N*-acetylglucosamine; QS, quorum sensing; RIP, RNAIII-inhibiting peptide; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

donor sodium nitroprusside (SNP) not only induces dispersal but also increases the activity of antimicrobial compounds, such as tobramycin, against established *P. aeruginosa* biofilms (352). Exposure to low levels of NO in *P. aeruginosa* biofilms induces genes involved in motility and energy metabolism and downregulation of adhesins and virulence factors (353). Notably, the chemotaxis transducer BdlA is involved in the NO-induced biofilm dispersal response.

(ii) Eradication of persisters. Another straightforward approach to fighting biofilms is to increase the activity of antibiotics against biofilm bacteria. As persister cells play a key role in the recalcitrance of biofilms toward antibiotics, the identification of compounds or associations that are active against persisters is important.

An adjuvant approach was recently proposed, based on the association of aminoglycosides and a sugar, such as mannitol or fructose, in order to increase antibiotic uptake and action against persister cells. After the sugar is taken up, it stimulates glycolysis, leading to the production of NADH, which, in turn, stimulates enzymes such as NADH dehydrogenases (NDH) or quinol oxidase. The electron transport chain oxidizes NADH and extrudes H^+ ions, thereby increasing the proton motive force (PMF). PMF, also called Δp, is composed of $\Delta\psi$ (the electrical potential across the membrane) and ΔpH (the transmembrane difference in H^+ concentration) (63, 354). In the case of mannitol or fructose, the generation of NADH stimulates PMF through an increase in $\Delta\psi$ (86). This stimulation of PMF leads to increased aminoglycoside uptake, and thus increased mortality of *E. coli* and *S. aureus* persisters *in vitro* and of *E. coli in vivo*, in a model of CAUTI. Recently, mannitol was also demonstrated to increase the aminoglycoside tobramycin's efficacy against *P. aeruginosa* biofilm persister cells, therefore pointing to a promising adjuvant with a broad range of activity (355).

Silver, usually used as an antimicrobial agent to coat material, was also proposed as an adjuvant to antibiotics against Gram-negative bacteria (356). It was demonstrated that silver could increase ROS production and membrane permeability and thus increase the effect of gentamicin, ofloxacin, or ampicillin against planktonic and biofilm persisters both *in vitro* and *in vivo* (356). It was also recently shown that persisters can be killed through the association of an antibiotic (rifampin) and a compound (ADEP4, an acyldepsipeptide) able to activate ClpP (88). The activation of ClpP through ADEP4 results in the degradation of more than 400 proteins. This association has been shown to successfully eradicate *in vitro* and *in vivo S. aureus* biofilms.

Finally, irrespective of the fact that the issue is currently being debated, several groups have tried to decrease persister tolerance through an increase in ROS production (121, 125, 126). Different potential targets have been identified by use of genome-scale metabolic models to predict ROS production (357).

(iii) Bacteriophages. The worldwide spread of multidrug-resistant bacteria and the shortage of new antibiotics are now leading to a revival of interest in phage therapy. Different authors have proposed the use of bacteriophages for eradication of biofilms.

One classical example is their use in lock therapy to treat catheter-related infections (237). Via this approach, microorganisms responsible for the infection should first be screened against a bank of phages so as to choose the phage strain associated with the greatest lytic capacity (237). Different phages have been used, including phage T4 against *E. coli* and phage F116 against *P. aeruginosa* (358–361). Note that some bacteriophages have also been reported to induce biofilm dispersal, such as PT-6, which induces *P. aeruginosa* alginase (362). The use of bacteriophages has also been proposed as a preventive measure, e.g., pretreated hydrogel-coated catheters with a cocktail of five *P. aeruginosa* bacteriophages were used in an *in vitro* model (363). Cocktails of phages are therefore a promising strategy for fighting biofilms. Recently, a cocktail of three phages was shown to reduce mouse ileum colonization by an O104:H4 enteroaggregative strain of *E. coli* (364). The onset of potential resistance and long-term innocuity must now be evaluated in order to validate these strategies.

In concluding this section, it is worth noting that many other compounds have been proposed or used to treat established biofilms, with as yet unknown mechanisms of action and untested clinical value. As a consequence, establishing a complete list of these antibiofilm, antibacterial, and sometimes immunomodulatory molecules is an arduous task (Table 2).

CONCLUDING REMARKS

Biofilm recalcitrance toward antibiotics is responsible for most of the difficulties encountered in the treatment of biofilm-related infections. Major advances have been made in the characterization of factors associated with this problematic biofilm property. Recognition of the role played by persister cells and the recent identification of several molecular mechanisms involved in the generation of persisters have already led to several potential antibiofilm treatments. Validation of these new approaches will likely require renewed interactions between fundamental research and clinical practice before these approaches can be included in future therapeutic arsenals for use against difficult-to-treat infections.

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