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REVIEW

Mechanisms of Bacterial Tolerance and Persistence in the Gastrointestinal and Respiratory Environments

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SUMMARY Pathogens that infect the gastrointestinal and respiratory tracts are subjected to intense pressure due to the environmental conditions of the surroundings. This pressure has led to the development of mechanisms of bacterial tolerance or persistence which enable microorganisms to survive in these locations. In this review, we analyze the general stress response (RpoS mediated), reactive oxygen species (ROS) tolerance, energy metabolism, drug efflux pumps, SOS response, quorum sensing (QS) bacterial communication, (p)ppGpp signaling, and toxin-antitoxin (TA) systems of pathogens, such as *Escherichia coli*, *Salmonella* spp., *Vibrio* spp., *Helicobacter* spp., *Campylobacter jejuni*, *Enterococcus* spp., *Shigella* spp., *Yersinia* spp., and *Clostridium difficile*, all of which inhabit the gastrointestinal tract. The following respiratory tract pathogens are also considered: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Burkholderia cenocepacia*, and *Mycobacterium tuberculosis*. Knowledge of the molecular mechanisms regulating the bacterial tolerance and persistence phenotypes is essential in the fight against multiresistant pathogens, as it will enable the identification of new targets for developing innovative anti-infective treatments.

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INTRODUCTION

The survival of bacteria is at least partly associated with the capacity of these microorganisms to detect and react to changes in environmental conditions. The machinery required to respond to environmental features is universally present in prokaryotic and eukaryotic cells. Several response mechanisms in bacteria are activated under stress conditions and controlled by proteins whose expression is associated with regulator genes. Interestingly, interactions between these mechanisms enable an efficient, coordinated response to multiple stressors.

Antimicrobial resistance is one of the main problems of the 21st century. The rapid spread of multidrug-resistant (MDR) pathogens has been described as a global crisis that may lead to an era without effective antibiotics (1). Failure of antibiotic treatment is typically attributed to resistance. However, it has long been realized that other mechanisms, such as tolerance and persistence, can also help bacteria to survive antibiotic exposure (2). Resistant bacterial populations (resistance phenotype) have the following three main characteristics: (i) the use of active, mutation-associated defense mechanisms to withstand drug-induced stress; (ii) growth of the surviving cells under drug pressure; and (iii) an inherited phenotype. The cellular changes that result as effects of the mutations include inactivation of antibiotics by increasing efflux, modifying targets, and directly modifying the antibiotic (3–5). Tolerant bacterial populations (tolerance phenotype) are bacterial populations which can outlive exposure to raised concentrations of an antibiotic, without any modification of the MIC, by slowing down essential bacterial processes. Tolerance may be acquired through exposure to environmental stress conditions (6) and applies only to bactericidal compounds (2, 5). Persister bacterial subpopulations (persistence phenotype) are persister cells that exhibit an epigenetic trait whereby they are tolerant to antibiotics but remain dormant and are not metabolically active (3). The following are characteristic of persistent subpopulations: (i) cessation of cellular activity (dormancy), (ii) no growth or change in concentration in the presence of drug, (iii) no inherited persistence phenotype, and (iv) cells revert quickly to wild-type growth once the drug pressure is eliminated and nutrients are administered (3). The relationships between resistant and tolerant populations and persistent subpopulations are complex (2, 7).

In this review, we use the definitions of resistant and tolerant populations and persistent subpopulations described by several authors (3, 8) (Fig. 1). These researchers followed the experimental evidence showing that persister cells do not grow (3, 9–13).

GASTROINTESTINAL AND RESPIRATORY ENVIRONMENTS

The environmental conditions in the gastrointestinal and respiratory environments differ in relation to their function in humans. Thus, the gastrointestinal environment is characterized by the presence of nutrients, gastric and pancreatic enzymes, bile salts, pH and temperature conditions, anaerobiosis, and bacterial competition. Moreover, the gut is the epicenter of antibiotic resistance (14).

On the other hand, the conditions in the respiratory environment are associated with its function, including variable levels of oxygen, nitrogen, carbon dioxide, and water vapor, pH and temperature conditions, external factors, and viral infections. The different characteristics largely determine which pathogens are capable of infecting these locations.

In this work, we analyzed the importance of the molecular machinery of tolerance and persistence in clinical pathogens that inhabit the gastrointestinal and respiratory environments. Gastrointestinal tract pathogens form part of the gastrointestinal microbiota (both commensal and opportunistic) (14) and include *Escherichia coli*, *Salmonella* spp., *Vibrio* spp., *Klebsiella* spp., *Helicobacter* spp., *Enterococcus* spp., *Campylobacter jejuni*, *Shigella* spp., *Yersinia* spp., and *Clostridium difficile*. Respiratory tract pathogens (both commensal and opportunistic) (15) include *Staphylococcus aureus*, *Pseudomonas*

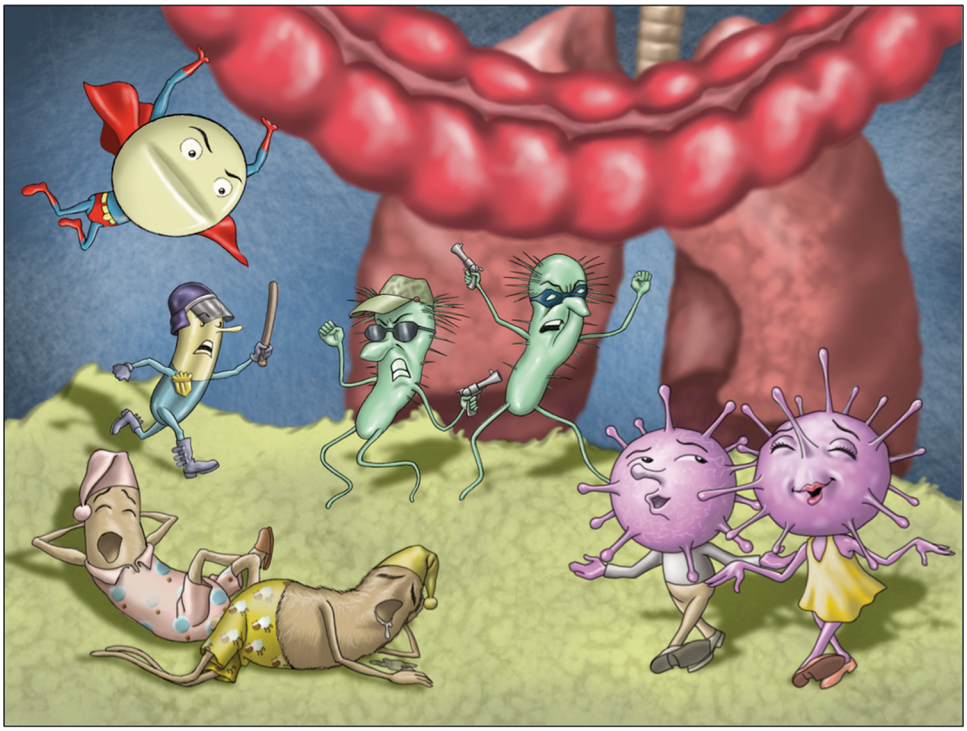

FIG 1 Illustration of resistant (green), tolerant (purple), and persistent (brown) subpopulations of bacteria interacting with the immune system (police) and antimicrobial treatment (superman) defense agents.

aeruginosa, *Acinetobacter baumannii*, *Burkholderia cenocepacia*, and *Mycobacterium tuberculosis*.

RELATIONSHIPS BETWEEN MOLECULAR MECHANISMS OF TOLERANCE AND PERSISTENCE

The molecular mechanisms involved in the formation of tolerant and/or persistent bacterial cells include the following: RpoS and the general stress response, oxidant tolerance (response to reactive oxygen species [ROS]), energy metabolism or drug efflux pumps, the SOS response, the quorum sensing (QS) system or bacterial communication, (p)ppGpp signaling, and toxin-antitoxin (TA) modules (7, 16, 17). In the following, we discuss these molecular mechanisms in gastrointestinal and respiratory bacteria.

General Stress Response (RpoS-Mediated Response)

RpoS and the other general stress responses are important molecular mechanisms whereby bacteria survive stress conditions (7). The *rpoS* gene encodes the sigma factor (S), which regulates the response (18) to conditions of stress, causing the accumulation of RpoS as cells enter the stationary phase and a rise in the number of related bacteria. RpoS-dependent gene expression leads to global bacterial stress resistance. Specific small RNAs (sRNAs) that encourage RpoS translation or the induction of antiadaptors that make this protein stable are induced in response to several stressors (19).

In isolates of the aforementioned gastrointestinal tract pathogens *E. coli* and *Salmonella enterica* serovar Typhimurium, RpoS and the general stress response have important roles in virulence, biofilm development, and bacterial survival. Under conditions of environmental stress or as cells enter the stationary phase, *E. coli* causes accumulation of RpoS (18, 19). RpoS mainly regulates genes and structural proteins associated with the formation and degradation of biofilms in response to stress (18). Several conditions can induce the general stress response in this pathogen, including nutrient deprivation, variations in temperature, biofilm production, high pH, oxidative

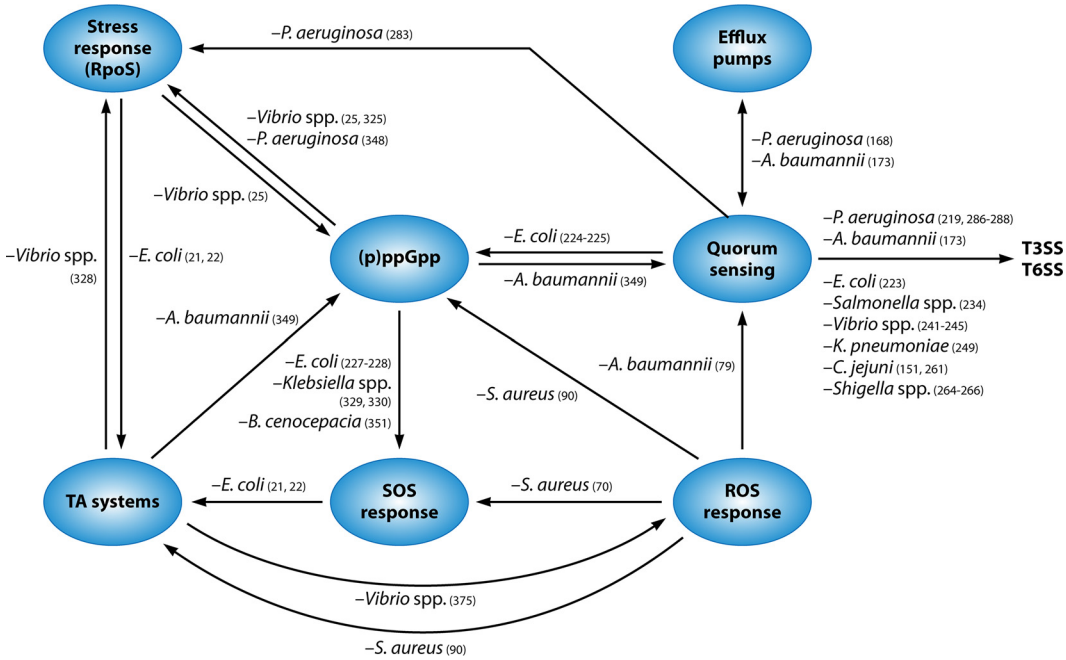

FIG 2 Links between the different mechanisms of tolerance and persistence described for gastrointestinal and respiratory pathogens.

stress, and other signals (20). In addition, the RpoS response and TA systems interact in *E. coli* (Fig. 2); for example, the antitoxins MqsA of the MqsR/MqsA TA module and DinJ of the YafQ/DinJ TA repress *rpoS* transcription and translation, respectively, until stress is encountered and the antitoxins are degraded (21, 22). In relation to *S.* Typhimurium, RpoS and the general stress response system also have a role in virulence or biofilm formation (23). RpoS levels are higher during the stationary phase or whenever bacteria are exposed to stress conditions (24).

On the other hand, when *Vibrio cholerae* colonizes the human gastrointestinal tract, RpoS regulates a system known as the "mucosal escape response." In this pathogen, RpoS expression is linked to an increase in the (p)ppGpp alarmone (Fig. 2), managing to improve motility and chemotaxis and presumably contributing to evasion of the mucosal response (25).

In clinical isolates of *Klebsiella pneumoniae*, a c-di-GMP phosphodiesterase protein controls the oxidative stress response and *in vivo* virulence, which is decreased by *rpoS* and/or by *soxRS* deletion, implying RpoS- or SoxRS-dependent control (26).

In *Shigella flexneri* and *Shigella boydii*, acid and base resistances are dependent on pH and are controlled by RpoS under stress conditions. For both types of resistance, the RpoS demand can be overcome by growth under anaerobic and moderately acidic conditions (27–30).

Interestingly, although RpoS is vital for the general stress response in numerous bacteria, it appears to be lacking in some pathogens, and it is not known whether other proteins carry out the same functions. *Helicobacter pylori* possesses alternative regulatory systems that were not observed until now because of the global response under stress conditions in isolates lacking the classic regulators. Proteins such as Fur and HspR compensate for the absence of RpoS (31). Three proteins associated with the stress response have been described for *Enterococcus faecalis*: (i) a general stress protein (gsp65), encoded by the hydroperoxide resistance *ohr* gene, which is induced in response to hydrogen peroxide, heat shock, acid pH, detergents, ethanol, sodium chloride, and *tert*-butylhydroperoxide (tBOOH) (32); (ii) the gsp62 protein, associated with the reaction to heat shock, acid pH, detergents (i.e., SDS or bile salts), ethanol, tBOOH, sodium chloride, and, to a lesser extent, hydrogen peroxide (33); and (iii) the

gls24 protein, implicated in resistance to bile salts and virulence (34–36). Nevertheless, the RpoS protein did not seem to have a role in expression of the genes associated with the pathogenesis of *Yersinia enterocolitica* infection; however, the RpoS protein was required by *Y. enterocolitica* grown at 37°C to survive a variety of types of environmental stress (37) and hostile environments (38). Analysis of the *C. jejuni* NCTC 11168 genome sequence demonstrated that RpoS is absent in this organism. In addition, in a study searching for an RpoS homologue in the NCTC 11351 strain of *C. jejuni*, the authors concluded that no such homologue was found (39). The absence of an RpoS homologue was confirmed for *C. jejuni* NCTC 11351 by the lack of induction of stress resistance in the stationary phase (39). The gastrointestinal pathogen *Clostridium* also lacks RpoS. Proteins such as HSP and those comprising the GroESL and DnaKJ systems have been associated with the reaction to chemical stress, whether from autologous metabolites or allogeneic toxic chemicals (e.g., derived carboxylic acids), high H^+ concentrations (low pH), antibiotics, or solvents (ethanol and butanol), all of which can have a major role in survival of the cells (40).

The important role of RpoS has also been described for respiratory pathogens, e.g., *P. aeruginosa*. In this bacterium, RpoS has been shown to positively affect the *pls* locus, which encodes enzymes that generate an extracellular polysaccharide involved in biofilm formation/expression (41).

However, other factors have been analyzed in pathogens such as *S. aureus* and *B. cenocepacia*. For *S. aureus*, the σ^B factor has been shown to be involved in the survival of cells under heat as well as electric and hydrostatic pressure (42). For *B. cenocepacia*, the importance of two sigma factors (other than RpoS), RpoN (AK34_313) and RpoE (AK34_2044), which are upregulated in the *fixLJ* deletion mutant relative to those in *Burkholderia* strain AU0158, has been studied. These sigma factors are critical for the survival of *B. cenocepacia* inside macrophages, and RpoN has been found to be essential for biofilm production (43–45).

Oxidant Tolerance (ROS Response)

Reactive oxygen species (ROS) are chemically reactive chemical species with oxygen (hydrogen peroxide [H_2O_2], superoxide [O^{2-}], and hydroxyl radical [OH^-]). In a biological context, ROS are produced as a natural response to the normal metabolism of oxygen and have important functions in cell signaling and homeostasis. Nevertheless, during times of environmental pressure (e.g., UV, heat, or drug exposure), ROS levels can rise. This can produce DNA damage, lipids, and proteins that cause cell death (46–48). Superoxide dismutase (SOD) and catalase enzymes or other antioxidant agents, such as glutathione and vitamin C, can eliminate ROS. When an imbalance between the mechanisms of production and elimination of ROS occurs, with an increase in the former, cells are subjected to oxidative stress (49).

In drug-tolerant *E. coli* cells, SOD and catalase have been shown to have a protective function (50). In other gastrointestinal pathogens, such as *S.* Typhimurium, many genes must be expressed in order to inactivate ROS, in a process controlled by regulons, such as SoxRS, OxyR, σS, σE, SlyA, and RecA, as well as the Dps protein, which halts bacterial growth under the control of the σ factor RpoS (51) (Fig. 2). The ROS response favors intestinal inflammation, thus allowing this microorganism to spread in the gut (52).

Interestingly, *V. cholerae* can generate two types of catalases, KatB and KatG, to promote ROS homeostasis (53). Moreover, in *V. cholerae*, the transcriptional regulator OxyR is critical for antioxidant defense and enables the microorganism to scavenge environmental ROS to facilitate population growth (54). For this pathogen, the importance of the role of the ROS response in mediating the cholix toxin, a virulence factor that displays the action of ADP-ribosyltransferase on eukaryotic elongation factor 2 of host cells and leads to cell death, was recently demonstrated (55).

On the other hand, *K. pneumoniae* pyogenic abscess isolates often contain heavy capsular polysaccharides (CPS) and escape phagocytosis or death due to the action of serum factors (56, 57), oxidative stress, and the ROS response (58, 59). The thick, viscous

CPS also control bacterial colonization and biofilm production at the infection location (60).

H. pylori induces chronic inflammation of the stomach epithelium and evades clearance via numerous factors, such as adhesion, cell motility, and detoxification of ROS and toxins (61). All *H. pylori* strains encode catalase and SOD proteins to detoxify ROS, and *H. pylori* arginase limits NO production via macrophage-, neutrophil- and epithelial cell-derived nitric oxide synthase (62, 63).

Characterization of ROS detoxification enzymes, such as KatA, SodB, AhpC, Tpx, and Bcp, in *C. jejuni* has demonstrated the value of these cellular defense systems for the survival of this pathogen against ROS (64). During host colonization, *C. jejuni* is subjected to damage caused by ROS produced by the host immune system and the gut microbiota. However, *C. jejuni* possesses important ROS detoxification methods that allow it to outlive and colonize the host (64).

Some interesting features of the ROS response have also been described for other gastrointestinal bacteria, such as *Shigella dysenteriae*, *Yersinia enterocolitica*, and *Clostridium difficile*. The *Shigella dysenteriae* 1 toxin produces intestinal infection via a decrease in the endogenous intestinal protection against ROS (65). Two new SODs associated with survival in acidic environments, including that in intraphagocytic vesicles, have been described for *Yersinia enterocolitica* (66). The siderophore yersiniabactin increases the virulence of *Y. enterocolitica* and blocks the development of the ROS response through eukaryotic cells (leukocytes, monocytes, and macrophages) (67). Finally, in a study of the distribution of the main enzymes in *Clostridium* involved in antioxidative defense, i.e., SOD and catalase, the physiological responses (induction of SOD and catalase) to factors triggering oxidative stress in the cells of strict anaerobes were shown to be responsible for the ability of the bacteria to remain viable under aerobic conditions (68). Moreover, studies of the induction of ROS by TcdA and TcdB toxins have dissected pathways contributing to this event, and there is speculation about the role of ROS in mediating pathogenesis (69). *Clostridium difficile* manages oxidative stress efficiently, and survival of this anaerobic microorganism is therefore consistent with ROS being mediated by TcdA and TcdB and thus favoring inflammation (69). On the other hand, the enzyme glutamate dehydrogenase (essential for growth of *C. difficile*) participates in the production of alpha-ketoglutarate, which contributes to H_2O_2 tolerance associated with the ROS response (69).

It was recently demonstrated that heterogeneous respiratory bacteria, such as methicillin-resistant *S. aureus* (MRSA) strains, can exist as two populations, with a heteroresistant phenotype (HeR-MRSA) and a homoresistant phenotype (HoR-MRSA), which are induced by β-lactam antibiotics via oxidative stress and mediated by the ROS response and DNA damage (Fig. 2) (70). The production of ROS during β-lactam treatment seems to be regulated by catalases and dismutases (SODs), which protect organisms with the heteroresistant phenotype from cell death and encourage cell survival. Moreover, disabling the tricarboxylic acid (TCA) cycle activity has a negative effect on ROS production, showing the role of metabolic modifications in the homoresistant phenotype (70). Finally, mutagenesis stimulated by β-lactams in cells with the heteroresistant phenotype demonstrates the conjunction of ROS production and the SOS-induced response (70). On the other hand, conservation of the persistent state in biofilms produced by several pathogens has been associated with adaptation of metabolic processes, mainly the processes associated with ROS formation and the TCA cycle. In addition to the characteristics of cells associated with biofilm production, metabolic changes are also important, as confirmed by survival studies with planktonic *S. aureus* cells. The appearance of mutants without the TCA cycle enzymes succinate dehydrogenase and aconitase suggests an improved level of survival in the stationary phase (71, 72). The decrease in ROS formation was determined to be a fundamental characteristic at this point. The ROS response appears to be associated with programmed cell death in *S. aureus* strains (73).

SOD and catalase have been analyzed in *P. aeruginosa*, and the *sodB* gene has been connected with an important protective role against UV-C radiation (74). The same

researchers also found that levels of the POX enzyme increased under stress tolerance (74). According to *in vitro* evidence, the environmental factors that trigger the selection of mucoid colonies are oxidative stress, low oxygen concentrations, and high osmolarity, i.e., conditions commonly found in the lungs of infected patients. Alginate overproduction confers resistance to phagocytosis but also appears to increase the sensitivity toward β-lactams and other types of antibiotics (75, 76) and contributes to the persistent inflammation of the airways (77). The parameters associated with mucoid transition in patients include persistence of bacteria in the sputum and the use of inhaled bronchodilators and inhaled antibiotics, such as colistin (78). A connection between the QS system and the expression of catalase and dismutase proteins in the ROS response of this pathogen has been described (Fig. 2) (79).

Four catalases (KatA, KatE, KatG, and KatX) have been described for *Acinetobacter* spp., among which KatE is the most effective in the resistance to H_2O_2 (80). In total, 107 differentially expressed proteins have been identified in *Acinetobacter baumannii* in relation to oxidative stress and are mainly involved in signaling, supposed virulence factors, and the stress response (including oxidative tolerance) (81). Interestingly, oxidant-tolerant cells of this pathogen showed a higher survival rate in response to several bactericidal antibiotics (41, 81).

Persistence is decreased in *B. cenocepacia* mutants lacking catalase or SOD proteins (82). The results obtained by Van Acker and colleagues have contributed greatly to our comprehension of the molecular machinery controlling antimicrobial tolerance in biofilms formed by the *Burkholderia cepacia* complex, revealing that these biofilms carry tolerant and persistent cells (83). The TCA cycle was downregulated in the remaining persistent cells, which thus prevented ROS production (detoxification). At the same time, the persistent cells switched on a different pathway, the glyoxylate shunt, which may become a new target for therapy. The aforementioned study also demonstrated that treatment of *B. cenocepacia* with tobramycin induces ROS production and that persister cells depend on ROS detoxification mechanisms, while processes responsible for ROS production (e.g., the TCA cycle, processes resulting in the production of NAD or flavin adenine dinucleotide, and the electron transport chain) are downregulated in persister cells (83).

Mycobacterial persister development under isoniazid pressure was related to the stochastic difference in the pulsatory expression of KatG, a catalase peroxidase needed for processing and activation of the drug. Slow-pulsing cells processed smaller amounts of the drug and therefore survived longer than fast-pulsing bacteria (84). The persister subpopulation of mycobacteria shows a different type of sensitivity to hydroxyl radicals due to their tolerance to antimicrobials, unlike the larger population, which is susceptible to them. There is increasing agreement on the importance of ROS and oxidative damage in antimicrobial tolerance in other populations resistant to antimicrobial elimination, and the crucial function of ROS in a stochastic persistent subpopulation pattern has been demonstrated for this pathogen (85). However, stress promotes other metabolic pathways in mycobacteria, leading to reduced levels of ROS and increasing the limit for antibiotic-mediated death (86).

Finally, several compounds induce the ROS response in gastrointestinal pathogens, leading to the development of tolerant populations and thus favoring colonization and bacterial pathogenesis, as follows. (i) Salicylate can induce tolerance in *E. coli* by generation of ROS. Salicylate-induced ROS cause a decrease in the membrane potential, reduce metabolism, and lead to increased tolerance of ROS (87). (ii) Iron is important for pathogenic bacteria, such as *S.* Typhimurium. Free cytoplasmic iron is used in the formation of radicals and can stimulate the antimicrobial actions of ROS. It has been shown that mice with deficient levels of this metal have a lower risk of developing *S.* Typhimurium infection (88). (iii) Although *Salmonella* does not produce indole, when it uses the indole produced by other bacteria, its tolerance of antibiotics increases (52). (iv) Antibiotics such as vancomycin and penicillin may have an impact. The dependence of *Enterococcus* spp. on SOD for tolerance of vancomycin and penicillin is usual for antimicrobial-susceptible enterococci (89). (v) A demonstration of the response to

ethanol-induced stress (EIS) in *S. aureus* strains was provided by the expression of 1,091 genes, of which 291 were upregulated (90). The EIS caused upregulation of genes that promote stress response networks (90), including the ROS response, (p)ppGpp, and TA modules (Fig. 2). The transcriptional profile of MRSA pathogens indicated that they reacted to EIS by entering a state of dormancy and modifying the expression of elements from cross-protective stress response systems in an effort to preserve the preexisting proteins (90).

Energy Metabolism

Regarding energy metabolism, we highlight two mechanisms in relation to tolerant populations. First, cytochrome *bd* is a prokaryotic respiratory quinol:O_2 oxidoreductase that enhances the tolerance of cells to oxidative stress (ROS response) and nitrosative stress conditions. When aerobic metabolism is restricted by oxygen limitation in the cells, the cytochrome *d* complex (encoded by the CydAB operon), one of the terminal oxidases in the respiratory network, predominates (91, 92). Overexpression of this operon has been implicated in chlorhexidine (biocide)-tolerant cells (93, 94) and in taurine metabolism (93).

Cytochrome *bd* complex. The cytochrome *bd* complex (encoded by the CydAB operon) is the main element in the respiratory chain when aerobic metabolism is restricted by oxygen limitation (95). This mechanism has been analyzed in gastrointestinal bacteria, such as *E. coli* (95) and *S.* Typhimurium (96). When *S.* Typhimurium enters host tissue, it is subjected to an oxygen partial pressure (pO_2) of 23 to 70 mm Hg (3% to 10% oxygen), which is significantly lower than the atmospheric pO_2 of 160 mm Hg (21% oxygen) (97). The survival of *S.* Typhimurium in tissues during infection of mice is due to the presence of high-affinity cytochrome *bd* oxidase (98). Cytochrome *bd*-II produces an increase in epithelial oxygenation, which together with nitrate respiration drives expansion of this pathogen within the gut lumen (96, 99). This pathogenic strategy is acerbated by oral antibiotic therapy, since it enhances *Clostridium* depletion, which may clarify why treatment with oral antibiotics often fosters infection with antibiotic-sensitive *S. enterica* serovars producing human gastroenteritis (100). The cytochrome *c* peroxidases were also similar in *E. coli* strains described as H_2O_2 degraders and in anoxic *Salmonella* spp. (101).

The *V. cholerae* genome encodes four different respiratory oxygen reductases under limiting conditions, depending on the gastrointestinal localization (102). Three of these use ubiquinol as a natural electron donor (103), and the fourth (cbb_3-type heme-copper oxygen reductase) (104) uses cytochrome *c* (105).

Little is known about the energy metabolism (i.e., cytochrome *bd*) in strains of *Klebsiella pneumoniae*. Nevertheless, the ability of *K. pneumoniae* to grow anaerobically with citrate as the unique carbon source is known (106). The presence in *K. pneumoniae* of the genes responsible for citrate fermentation has been described for a group of 13 kb (107), in which variation in several isolates helps with adaptation to nutritional characteristics (108).

A cytochrome *bd*-type oxidase has been described for the *H. pylori* H_2-oxidizing membrane-associated respiratory chain (109), and differences relative to other pathogens have been reported (110). The possible relationship between the development of duodenal ulcers caused by *H. pylori* and its capacity to survive in the presence of bile acids conjugated with taurine has been contemplated (111).

Some authors have detected the presence in *C. jejuni* of a low-affinity oxidase resistant to cyanide, belonging to another type of cytochrome (112).

Functional studies of the cytochrome *bd*-type enzyme in *E. faecalis* V583 have been carried out (113). Interestingly, in relation to energy metabolism, the Clp ATP-dependent protease operon, implicated in stress survival, is highly conserved and enables growth at high temperatures in Gram-positive bacteria, including *Enterococcus faecalis* (114).

Cytochrome *bd* expression has been associated with normal intracellular survival and virulence in *S. flexneri* (115). Moreover, in a study involving an *S. flexneri cydC* strain

lacking cytochrome *bd*, the bacteria were quickly cleared from the lungs of intranasally inoculated mice (116).

S. aureus and *P. aeruginosa* cause opportunistic infections and regularly infect the lungs of cystic fibrosis (CF) patients. *S. aureus* has the capacity to resist the action of pyocyanin and hydrogen cyanide, which are small respiratory inhibitors, because of the action of the cytochrome *bd* quinol oxidase (117). The cbb_3-type cytochrome oxidase subunit (which catalyzes the final stage in respiration, displaying a strong affinity for oxygen) supports *P. aeruginosa* biofilm growth and bacterial pathogenesis (118). Hence, cbb_3-type cytochrome oxidases may be important therapeutic targets (118). On the other hand, elevated quantities of the second messenger cyclic di-GMP (cdG), produced by several mutations, are linked to overproduction of Pel and Pls exopolysaccharides and fimbrial adhesins in *P. aeruginosa* and are consequently involved in small-colony variant (SCV) formation (119–122). In addition to the cdG signaling pathways, SCV generation is influenced by other regulatory systems, such as the components of the GacAS mechanism, in which GacA regulates the response and GacS is the phosphorylating transmembrane histidine protein kinase. This system controls the RsmAZY regulatory system, and the combined regulatory network influences the transition between acute and chronic infectious lifestyles of *P. aeruginosa*. Mechanistically, phosphorylated GacA fosters the transcription of RsmY, sRNAs, and RsmZ genes, thus blocking the activity of RsmA by participating in its binding and attenuating the inhibitory effects on the target mRNAs, including those related to the synthesis of the Psl exopolysaccharide (123, 124). The SCV phenotype is promoted by deletion of the *rsmA* gene in *P. aeruginosa* strain PAO1 (41). The deletion also increases infectious persistence in mouse lung infections (125). Moreover, mechanisms of specific tolerance, such as the production of periplasmic glucan molecules, which inactivate aminoglycosides, are preferentially expressed in *P. aeruginosa* biofilms rather than in planktonic cells (126, 127). Together these mechanisms promote the prevalence of *P. aeruginosa* in the airways of infected patients.

In *Acinetobacter calcoaceticus*, quinoprotein glucose dehydrogenase interacts with the *b*-type cytochrome(s) and with cytochrome *o* and cytochrome *d* (both cytochrome oxidases) under stress conditions (128).

Finally, in *M. tuberculosis*, the *phoU* gene controls phosphate uptake, thus regulating the *pst* operon. The *phoU* gene is well conserved among bacteria and has homologues in *M. tuberculosis*. Mutants of *M. tuberculosis* are influenced by the *phoY2* gene, displaying low persistence both *in vitro* and *in vivo* (129–131).

Tau metabolism. In relation to Tau metabolism, cysteine or sulfate deficiency leads to *tauD* (or an orthologue) being essential for growth of *E. coli* (132, 133). The presence of TauD leads to the production of sulfite for use as a source of sulfur by catalyzing the hydroxylation of taurine alpha-(2-aminoethanesulfonic acid) in the sulfonate. Several studies have focused on *E. coli* TauD, which is an important member of the huge, universal family of αKG-dependent nonheme iron oxygenases (134, 135).

Some studies have also been conducted with *V. cholerae* in relation to activation of the virulence factor associated with the metabolism of taurine and with bile salts, favoring colonization of the gastrointestinal tract (136–139). Studies of the role of taurine (enzymes and transporters) associated with sulfate metabolism in this pathogen are scarce.

However, when *H. pylori* colonizes the tissue, bile has been observed to influence cell gastric epithelial kinetics, promoting gastric cancer (140). Interestingly, bile chemotactic gradients (mainly taurocholic and taurodeoxycholic acids) across the gastric mucus layer may therefore contribute to directing *H. pylori* to the pyloric antrum and thus to enabling this important pathogen to attain high population densities on the mucous layer in the area of the gastric epithelium (141).

Taurine is another important source of energy in *S. aureus*, and CymR has been considered an important regulator of cysteine and taurine metabolism participating in biofilm formation (142).

Moreover, the important functional role of the *tauRXYPI* cluster, implicated in taurine metabolism in *A. calcoaceticus*, has been analyzed under nitrogen limitation conditions

(143). The inhibitory effect of taurine on the formation of biofilm during alkane degradation was recently studied and shows that taurine probably affects the alkane-induced cell surface (144).

Finally, in *B. cenocepacia*, regulation of sulfur as an environmental source of energy has been associated with the *tauABC* operon (145).

Efflux Pumps

Efflux pumps are needed to eliminate toxic elements or to maintain the balance of compounds that are vital for bacterial survival (146). Various researchers recently demonstrated that increased expression of efflux systems is vital for active maintenance of a low intracellular antibiotic concentration, and thus specifically the persister state, in nongrowing, nondividing *E. coli* cells (147–149). Moreover, an active mechanism can also decrease the concentration of the toxic compound (drug), which raises the drug MIC and thus appears in populations with mixed resistance and tolerance phenotypes (5). The multidrug efflux pumps that participate in tolerance and resistance processes can be upregulated by several signals, such as oxidative stress (ROS response) and QS systems, including the type III secretion system (T3SS), T6SS, and other virulence factors (Fig. 2) (150, 151).

In vitro studies with *E. coli* have demonstrated that paraquat-induced tolerance is reliant on the AcrAB multidrug efflux machinery (152). Moreover, overexpression of this efflux pump was shown to be vital for the active maintenance of a low intracellular antibiotic concentration, and thus the tolerant persister state, specifically in nongrowing, nondividing *E. coli* cells, suggesting the coregulation of dormancy and active processes for the persistence phenotype (147, 153). In addition to the AcrAB system, *Salmonella* possesses at least 11 multidrug efflux pumps (11, 154). Among them, we highlight the following. (i) We first mention the AcrD efflux pump of *S.* Typhimurium (57). After inactivation of this pump, variations of expression of several genes involved in metabolism, stress responses, and virulence were detected. For example, a reduction in the expression of genes involved in pathogenesis or those that encode products of metabolism of tricarboxylic acid and purines was observed. The exact same effect was observed for the expression of virulence genes in *Salmonella* pathogenicity islands (SPI-1, SPI-2, SPI-3, SPI-10, and SPI-18). Levels of fumarate associated with swarming motility were also altered after inactivation of the AcrD efflux pump (11). (ii) The MdtD pump stimulates the efflux of citrate, an iron chelator generated during aerobic metabolism. When this efflux pump is induced by stress, iron is expelled from the cell, thus reducing bacterial growth. Expression of a citrate transporter (IceT) leads to a decrease in the vulnerability to ROS, nitrosative stress, and antimicrobial agents. Stress resistance and antibiotic tolerance are mediated by this protein (IceT) via regulation of metabolism, redox chemistry, and intracellular iron (88). (iii) Finally, in the presence of H_2O_2, the MacAB drug efflux pump protects *S.* Typhimurium against ROS by inducing the formation of a compound that confers resistance to extracellular H_2O_2. Another function of this protein is to facilitate the growth of *S.* Typhimurium within macrophages (154, 155). The functions of efflux pumps in multiresistance and in tolerance and/or persistence under stress conditions are not known for this pathogen.

A new multidrug efflux pump, EmrD-3, was recently discovered in *V. cholerae* O395 (156).

In *K. pneumoniae*, the AcrAB efflux pump promotes the development of pneumonia by acting as a virulence agent that fights the innate immune defense in the lung (157). Extreme virulence of *K. pneumoniae* isolates was recently found to be associated with increased expression of the AcrAB and OqxAB efflux pumps (158).

In *H. pylori*, an association between biofilm formation and loss of sensitivity to antibiotics was also detected through the action of two expulsion pumps in the process (159).

A contribution of the Cme ABC efflux pump to antibiotic resistance in isolates of *Campylobacter jejuni* has been reported (160). In addition, the *cmeA* gene, which encodes a multidrug efflux transporter, is upregulated in the presence of deoxycholic

acid in *C. jejuni* wild-type strains relative to that in T6SS-deficient strains, suggesting a relationship between the suppression of proliferation and the role of this efflux pump, regulated by the T6SS (151). The relevance of the T6SS to the adhesion, invasion, and colonization of the host and also to the adaptation to deoxycholic acid was thus confirmed, demonstrating the key role of this system in the pathogenesis of *C. jejuni* (151).

Researchers in South Africa recently considered drug resistance, efflux pumps, and virulence characteristics in different species of *Enterococcus* that occur in surface waters (161). Most of the isolates had four efflux pump genes (*mefA*, *tetK*, *tetL*, and *msrC*) and virulence genes, such as the *asa1*, *cylA*, *gel*, and *hyl* genes (161).

Moreover, for *Shigella*, we highlight two efflux pumps, as follows. (i) The AcrAB efflux pump has been connected to resistance to bile salts as well as to survival and pathogenicity during host transit and later gastrointestinal infection (162). (ii) The MdtJI efflux pump, which is involved in the extrusion of toxic compounds and allows survival of bacteria within infected macrophages, has been described for *Shigella* (163).

Regarding the transporters, we can highlight the RosA/RosB efflux pump of *Yersinia enterocolitica* (164). For this pathogen, resistance to new cationic antimicrobial peptides has been described as being due to an ejection pump/potassium antiporter mechanism consisting of the RosA and RosB proteins (164).

Finally, CdeA, a multidrug efflux pump belonging to the MATE family and involved in Na^+ transport, was identified in *Clostridium difficile* but has not been found to be associated with antibiotic resistance (165).

In respiratory bacteria, such as *S. aureus*, the expression of the Qac efflux pumps has been associated with increased tolerance to biocides (166). The predominant antibiotic resistance mechanism in *P. aeruginosa* CF isolates is the overexpression of efflux pumps, particularly those belonging to the RND superfamily. *P. aeruginosa* has genes for at least 12 of these systems, although we can highlight MexAB, which is involved in tolerance to colistin and biofilm formation (167). Moreover, this efflux pump is associated with the transport of 3-oxo-acyl-homoserine lactones, which are the signals used in cell-to-cell communication (QS) (168). The capacity of *P. aeruginosa* infections to persist in the lung regardless of antimicrobial treatment depends on the intrinsic tolerance of the bacterium to antibiotics and the readily acquired resistance to new drugs (169).

In *Acinetobacter baumannii*, increased biofilm production is associated with overexpression of two efflux pumps: AdeABC and AdeFGH (170–172). Under bile salt pressure, *A. baumannii* strain ATCC 17978 and *A. baumannii* clone ST79/PFGE-HUI-1 (a clinical strain lacking the AdeABC efflux pump) overexpress the glutamate/aspartate transporter as well as virulence components associated with activation of the QS system (biofilm, surface motility, and T6SS components) (173).

RND efflux pumps, such as BCAM1945-1947 (RND-9) and BCAM0925 (RND-8), protect the biofilm complex of *B. cenocepacia* from tobramycin, while the BCAL1672-1676 (RND-3) pump is important for resistance of biofilms to ciprofloxacin and tobramycin (174).

Finally, little information is available about efflux pumps in *M. tuberculosis*.

SOS Response

The SOS response is triggered by DNA damage, allowing repair of genetic material to enhance cell survival. The SOS system can be considered an important mechanism of bacterial survival under stress conditions, which are related to other stress responses (7, 50, 175). The SOS response involves genes that not only affect cellular processes, such as DNA recombination and repair, but also affect pathogenesis, antimicrobial resistance, and biofilm production (176). The proteins that make up the SOS system include a transcriptional repressor called LexA and a DNA-binding activating protein, RecA (177). However, other proteins may be involved.

As mentioned above, the RecA protein positively regulates the SOS system in *E. coli* (178, 179). The SOS system contributes to DNA repair but also induces the development of the type I TA module toxin TisB in a subpopulation of *E. coli* (180). Different studies

have demonstrated that under these conditions (including treatment with fluoroquinolones), the SOS response encourages the production of persister cells through positive regulation of the expression of the TisB toxin in *E. coli* (152, 175, 181). In *Salmonella* spp., swarming motility, bacterial virulence, and antibiotic tolerance are related, and the bacteria are able to control their motility when DNA damage is present, by means of the SOS induction system (RecA) (176). The lack of the RecA protein in *Salmonella enterica* impairs the swarming ability and also reduces the capacity of the bacterium to cross the intestinal epithelium (182–184).

Stress response pathways that are crucial for the development and survival of *V. cholerae* persister cells, especially the SOS system (RecA), have been found to encourage horizontal gene transfer between microorganisms, thus enhancing resistance (185). Antibiotics such as quinolones activate the SOS response and thus increase the incidence of horizontal transfer in *V. cholerae* (177). Moreover, this pathogen responds to other antimicrobials, such as aminoglycosides, chloramphenicol, and tetracycline, by stimulating the SOS response (175).

Interestingly, several canonical heat and cold shock proteins in *K. pneumoniae* strains are upregulated under extreme temperatures (low [20°C] and high [50°C]). Among these proteins, we highlight RecA, although other proteins (GrapE, ClpX, and DeaD) may be involved in the response (186).

DNA damage repair in *in vivo* colonization by *H. pylori* requires enzymes, such as RecA and AddAB, which are involved in survival at low pH (187, 188).

The RecA protein has also been characterized in relation to DNA damage repair in *Campylobacter jejuni* (189). On the other hand, plasmids encoding UmuDC-like proteins with SOS function have been located in isolates of *Streptococcus pneumoniae* Rx1 and *E. faecalis* UV202 (190).

SOS proteins, such as topoisomerases, and histone-like DNA binding proteins, such as H-NS, HU, and IFH, which are essential for maintaining bacterial DNA organization, have been investigated in *Shigella* spp. (191). The involvement of H-NS in suppressing DNA repair in *Shigella* spp. after UV irradiation has been described (191). The role of H-NS in *Yersinia enterocolitica* has also been reported (192).

Finally, the features of the SOS response have been studied in *C. difficile*, and the authors reached the conclusion that the SOS system is related to *C. difficile* sporulation and that the induction of the SOS system can stimulate biofilm production in this pathogen (193). The same authors also showed that LexA controls the expression of toxin genes, metronidazole resistance, biofilm production, and sporulation (193). The relationship between sporulation and the SOS system was also found in *Bacillus subtilis*, through the *sda* gene (193). Although this gene is absent in *C. difficile* (194), Walter and colleagues studied how LexA interacted *in vitro* with the promoter region of another gene associated with sporulation, *sspB* (194).

The proportion of *S. aureus* mutants (i.e., SCVs that are very tolerant to antimicrobials and that can survive in host cells) is higher in cultures exposed to fluoroquinolones and mitomycin C than in nonexposed cultures, and this was found to be correlated with a larger proportion of mutations (indicated by resistance to rifampin) and followed by stimulation of the SOS DNA damage response (195). These findings indicate that environmental stimulation (e.g., with antimicrobial agents that lower replication fidelity) increases the formation of SCVs by activating the SOS response and thus boosts difficult-to-treat persistent infections (195). It has been found that *Staphylococcus aureus* strains can adapt to oxidative stress via a mechanism that produces subpopulations of H_2O_2-resistant SCVs with improved catalase production (196). This occurs through a mutagenic DNA repair pathway that includes RexAB, RecA, and polymerase V (Pol V) (196). The SOS response is more complex in *P. aeruginosa* than in *E. coli* due to the participation of two other chromosomal regulators (PrtR and PA0906), which are present in most *P. aeruginosa* isolates and other species of the genus, in addition to LexA (197–202). In *P. aeruginosa*, DNA damage causes autocleavage of LexA by RecA and the elimination of repression of the LexA regulon, as in *E. coli* (198, 201, 203). The induction of PrtR-managed genes adversely affects survival during genotoxic stress,

decreases antimicrobial resistance, and reduces resistance to oxidant agents (204). In addition, the UmuDpR protein has been demonstrated to suppress expression of *P. aeruginosa* SOS genes regulated by LexA (205).

Regarding *A. baumannii* and *Acinetobacter baylyi*, several authors have examined the role of the RecA protein and UmuC proteins in repairing DNA damage, and therefore in the cellular defense against pressures produced by agents that damage DNA, antibiotics of various families (ciprofloxacin and tetracycline) (206), and oxidizing elements (207–209). Hemolytic Acb strains have been shown to be significantly more tolerant to UV treatment than nonhemolytic isolates and those belonging to other *Acinetobacter* species. This demonstrates the diversity of SOS responses in *Acinetobacter* strains and may partly explain the emergence of *A. baumannii* and *Acinetobacter ursingii* (210). Moreover, a DNA damage-inducible response has been described and found to promote drug resistance in *A. baumannii*, especially in stressful environments (211).

Little is known about the role of the SOS response in *B. cenocepacia*.

Finally, for *M. tuberculosis*, a regulon including Y-family DNA polymerases (ImuA′ and ImuB), which contributes greatly to damage tolerance (in conjunction with the C-family DNA polymerase DnaE2), has been described (212). Recently reported transcriptomic studies showed that various stress response regulons (e.g., the SOS response) and also different TA genes are positively regulated in *M. tuberculosis* persisters (213, 214). However, for this pathogen, an alternative RecA-independent DNA repair mechanism controlled by a ClpR-like factor has also been reported (215).

QS and Secretion Systems

QS is a bacterial communication network that allows cells to modify their collective behavior through signaling molecules, known as autoinducers, according to changes in the environment favoring the formation of persister cells (7, 216, 217). This process affects bacterial populations and determines the expression of genes regulating virulence, toxin production, motility, chemotaxis, biofilm production, and bacterial competition (secretion systems [T3SS and T6SS]) (Fig. 2), which may contribute to bacterial adaptation and colonization (218). In this review, we consider proteins from the QS system and secretion systems (T3SS and T6SS) due to their association with the development of persister cells, as described for pathogens such as *P. aeruginosa* and *Mycobacterium* spp. (219, 220). We hypothesize the importance in those pathogens of the relationship between the secretion systems (T3SS and T6SS) and the QS system regarding the development of persister cells. However, further studies of these secretion systems are required to clarify these relationships.

The QS systems described for *E. coli* include the LuxR homolog (SdiA receptor), LuxS (synthetase), autoinducer-2 (AI-2) and autoinducer-3 (AI-3) systems, and an indole-mediated signaling system (221). A QS system was related to the induction of *E. coli* persister cells through an indole molecule that overexpresses OxyR and phage shock regulons, preparing the subset of cells for future stress (222, 223). In contrast, other researchers have demonstrated that indole and indole analogs reduce persistence (224–226). Several researchers have investigated the connection between tolerance and persistence mechanisms and phenotypic factors controlled by the QS system. It has been shown that biofilms are often associated with intestinal infections caused by *E. coli* (18). As the main agent of urinary tract infections (UTIs), *E. coli* can form biofilms within the bladder epithelial cells and thus evade antibiotic activity (180). The bacterial growth rate determines the sensitivity to some antibiotics, as occurs with ciprofloxacin, and therefore the biofilm cells are protected from the action of these antibiotics when they grow at lower rates (20). Furthermore, biofilm formation in *E. coli* causes cells to have limited access to nutrients, thus increasing the levels of (p)ppGpp involved in tolerance to multiple drugs (Fig. 2) (227, 228). Finally, the QS system in enteropathogenic *E. coli* (EPEC) controls the activation of the type III secretion system, which participates in the modulation of virulence (229).

Three QS systems have been identified in *Salmonella*, all of which are formed by a synthase, a receptor, and a signal (221), as follows: (i) the unknown synthase, SdiA, and

3OC8HSL system, which has a motility function and promotes resistance to acids (230); (ii) the LuxS, LsrB, and AI-2 system, involved in expression of the Lsr gene cluster (uptake of AI-2) (231); and (iii) the QseB, QseC, and AI-3 system, which is involved in virulence features, motility ability, and biofilm production (232).

S. Typhimurium produces acyl-homoserine lactone (AHL) and the signaling molecule AI-2. Both molecules are produced and released mainly during exponential-phase growth and are implicated in biofilm formation (52). A recently published study showed that the presence of *in vitro* bile salts increases the killing of other bacteria by *S*. Typhimurium in the gut. This antibacterial activity is not efficient against all commensal bacteria. While *S*. Typhimurium outcompetes *Klebsiella oxytoca* or *Klebsiella variicola*, commensal species, such as *Enterococcus cloacae*, *Bacteroides fragilis*, *Bifidobacterium longum*, *Parabacteroides distasonis*, and *Prevotella copri*, are not overcome (233). Interestingly, *Salmonella enterica* and other pathogens present T6SS in association with T3SS, quorum sensing (QS), flagellum production, and QS regulators, which is essential for bacterial pathogenesis (234).

The different types of behavior controlled by QS in *Vibrio* spp., such as bioluminescence, T6SS and T3SS, biofilm production, and motility, make this bacterium an ideal model for studying quorum sensing. The following QS systems have been described for *V. cholerae*: (i) LuxS, LuxP, and AI-2; and (ii) CqsA, CqsS, and CAI-1. Both of these are related to biofilm production, extracellular polysaccharide formation, and other virulence agents (221, 235). QS systems are also involved in the *V. cholerae* persister phenotype (236). It is well known that *V. cholerae* can occur either as planktonic cells or adhered to a biofilm matrix that forms aggregates. As in other pathogens, biofilm production in *V. cholerae* is regulated by QS. Recent evidence suggests that biofilms are formed during the aquatic and intestinal phases of the *V. cholerae* life cycle and perform an essential function in environmental and intestinal survival and also in the transmission of infection (237). *V. cholerae* AlsR (quorum sensing-regulated activator) drives the expression of the acetoin gene cluster in response to glucose, acetate, or another activating signal (238). Furthermore, a model describing the regulation of the acetoin biosynthetic gene cluster by AlsR and AphA according to environmental conditions has been established for *V. cholerae* (238). In many species of *Vibrio*, the T3SS and T6SS are strongly associated with QS (239–242). *V. cholerae* uses the T6SS to compete with the different prokaryotic and eukaryotic cells that it finds in several environments and human hosts. Novel research on the expression of the T6SS indicates that this system may promote the persistence phenotype and the development of *V. cholerae* infection through direct opposition of bacterial competitors (243). *In vitro* studies have demonstrated that the *V. cholerae* T6SS is expressed by bacteriocins (mucins) and modulated by bile salts, which are modified by the microbiota (244). In relation to these findings, an intact T6SS is necessary for *V. cholerae* to become established in the guts of infant rabbits (245).

Interestingly, QS in *K. pneumoniae* is LuxS dependent, and AI-2 autoinducers (246, 247) participate in biofilm formation (248). In a study involving genomic extraction and data analysis for isolates of *K. pneumoniae*, three conserved regions were distinguished and found to contain T6SS genes, which are controlled by the QS system (249).

H. pylori generates extracellular signaling molecules associated with AI-2, which depends on LuxS function. In turn, this is determined by the growth phase, and production is higher in the mid-exponential phase (250–252). Its genetic variability and the capacity of *H. pylori* to develop biofilm, and thus to protect itself from environmental stressors, are responsible for the resistance of this pathogen to the usual treatments, and also for its persistence in human tissues (253). *In vitro* biofilm production by *H. pylori* is reflected in several studies (254–257). Moreover, this pathogen can even produce biofilms on the human gastric mucosa (257–260).

The LuxS-homologous protein which participates in the synthesis of AI-2 (a key participant in biofilm formation) has been located for *C. jejuni*. This bacterium can join to and produce biofilms on different surfaces (160). However, a *luxS* mutant did not show important changes in the virulence phenotype (CmeABC multidrug efflux pump,

cell morphology, or mucin penetration). As mentioned above, *C. jejuni* possesses an operational T6SS encoded by a complete T6SS gene cluster that forms part of an integration factor located in the genomes of some *C. jejuni* isolates (261). Moreover, the T6SS participates in tolerance of bile salts and deoxycholic acid (DCA) and in the pathogenicity characteristics of bacteria, such as adhesion and invasion (151).

E. faecalis readily forms biofilms. It is capable of acquiring resistance determinants, such as elements of the QS system (*fsrA*, *fsrC*, and *gelE*) and two glycosyltransferase genes (GTF genes), via horizontal gene transfer encoded by *epaI* and *epaOX*, which promote biofilm formation (262). Moreover, the *fsr* QS component and the GelE protease regulated by QS are involved in gentamicin, daptomycin, and linezolid resistance in *E. faecalis* biofilms but not planktonic cells (262).

Regarding *Shigella* spp., many authors have searched for multiple factors related to the QS system, including the production of the AI-2 signal, in *Shigella flexneri* (263). Moreover, the connection between T3SS and the QS activation process was described in 2011 (264, 265). More recently, it was shown that *Shigella sonnei*, but not *S. flexneri*, encodes a T6SS, providing a higher capacity for survival in the intestine (266).

The QS of *Yersinia enterocolitica* has also been investigated (267). Homologues of the LuxI (AHL synthase) and LuxR (response regulator) protein families have been analyzed for several species of *Yersinia*. Although *Y. enterocolitica* has a LuxRI pair (YenRI), other species have two pairs (267). Moreover, the same researchers demonstrated the role of QS in swimming- and swarming-type motilities of *Yersinia* (268). Other investigators have shown that biofilm formation may be inherent in *Y. enterocolitica*. The presence of biofilms greatly increased the minimum inhibitory concentration for bacterial regrowth (MICBR) for all antimicrobials (269).

In *Clostridium difficile*, QS plays a role in toxin synthesis (270) as well as biofilm formation (271). Along with LuxS and SpoOA, flagella and the cysteine protease Cwp84 are important for biofilm formation in *C. difficile* (271, 272).

In vivo experiments have examined the function of quorum detection systems in *Staphylococcus aureus* (SarA and Agr) in the development of persister cells in various types of infections (221). Agr has been shown to be associated with the formation of the persistence phenotype in *S. aureus* (273). Mutations of either *agrCA* or *agrD*, but not RNAIII, showed a rise in persister cell formation in stationary-phase cultures (273) (Fig. 3). In *S. aureus*, a connection between the modulation of AI-2 and different phenotypes displaying capsule formation, biofilm production, antibiotic resistance, and virulence has been observed (274–276). These findings have been corroborated in both laboratory experiments and animal models infected with *S. aureus*, in which *luxS* controlled biofilm formation by regulating the *icaR* locus. This regulator is a repressor of the *ica* operon (responsible for producing a polysaccharide composed of β-1,6-linked *N*-acetylglucosamine), which is necessary for biofilm formation (275). However, the function of LuxS in regulating the QS in *Staphylococcus* spp. remains controversial.

Quorum sensing in *P. aeruginosa* involves at least three functional QS circuits, two of which are controlled by *N*-acyl-homoserine lactone (HSL) signals (LasI/LasR and RhlI/RhiR) and the other of which is controlled by quinolones (221). Five signals have been identified: AI-2, *Pseudomonas* quinolone signal (PQS), autoinducer peptides (AIPs), AHLs, and diffusible signal factors (DSFs) (277, 278). This system is vital for the colonization and later survival of bacteria during infection, as it coordinates phenotypic changes, especially at the beginning of the infection and during binding to the host cell (279). Expression of QS genes is important in determining the progress of infection (acute or chronic). More than 10% of the genes in *P. aeruginosa* are controlled by QS, and all of these genes are associated with the production of virulence factors, as well as with biofilm formation, antibiotic resistance, surface motility, and stress response-produced adjustments of the metabolic routes (280–282). Moreover, the *rpoS* gene is known to control the Las system (Fig. 2), which is probably involved in the generation of tolerance to ofloxacin in *P. aeruginosa* (283). Small molecules spread in the environment (QS signals) can trigger biofilm disintegration. One of the most important factors contributing to the survival of *P. aeruginosa*, by generation of resistance or tolerance,

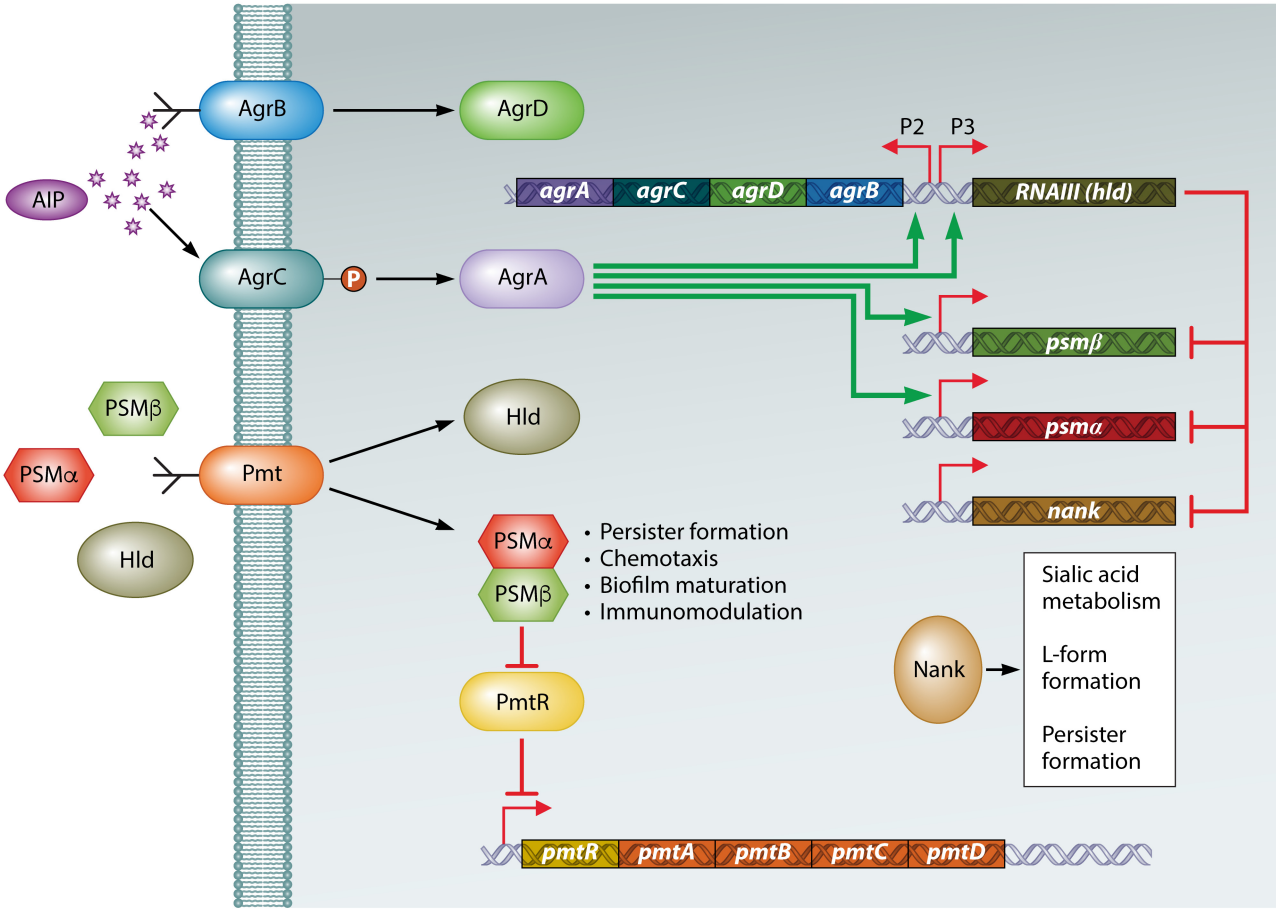

FIG 3 Role of the Agr quorum sensing regulatory network in the formation of persisters. The *agr* operon is activated by an autoinducer peptide (AIP) encoded by *agrD*, modified and transported by AgrB, and processed by AgrC (histidine kinase) and AgrA (response regulator). AgrA positively regulates P2 and P3 of the *agr* operon, activating AIP production from P2 and RNAIII from P3. In addition, AgrA promotes the expression of the *psm* genes, which encode phenol-soluble modulins (PSMs). The PSMs are transported by the Pmt system, encoded by the *pmt* operon. PSMs join PmtR (repressor of the *pmt* operon) to activate the production of PSM transporters. RNAIII negatively regulates the *psm* and *nank* genes. PSMs inhibit the formation of persisters, while Nank promotes their formation. (Adapted from reference 273.)

is the capacity to develop biofilms both *in vivo* and *in vitro*. Biofilms are more tolerant (up to 4 orders of magnitude) than planktonic cells to several antibiotics, as the antibiotics are less able to infiltrate the deepest parts of the structure and because the low oxygen and nutrient concentrations available to the bacteria at those locations render them metabolically inactive. Moreover, up to 1% of bacterial cells in biofilms are persister cells, which are dormant cells that are not affected by antibiotics (8). This type of cell is abundant in bacterial isolates from the lungs of chronic CF patients (284). In addition to mucoid colonies, SCVs, also known as dwarf colonies, are also commonly isolated from *P. aeruginosa* infections. SCVs are small (1 to 3 mm in diameter), form biofilms, attach strongly to surfaces, and display autoaggregative properties due to increased exopolysaccharide production (mainly Pel and Psl polysaccharides but sometimes also alginate) and high rates of production of pili (285). Moreover, these SCVs are usually nonmotile and resistant to several different classes of antibiotics (285). *In vitro* tests have shown that exposure to sublethal concentrations of antibiotics, such as aminoglycosides, selects for the formation of SCVs; hence, exposure to antibiotics may also trigger the selection of SCVs *in vivo*. In CF patients, prolonged persistent infections, deterioration of pulmonary function, and increased antibiotic resistance are all correlated with the presence of SCVs in sputum. For *Pseudomona aeruginosa*, the regulators and growth states involved in H2- or H3-T6SS expression, including

quorum sensing and iron depletion, have been analyzed (286, 287). Analysis of RsmA-mRNA complexes of *P. aeruginosa* by gel shift assay as well as translational and transcriptional fusion led to the identification of 40 genes, distributed in six operons, which are translationally controlled by RsmA (288). RsmA is a negative regulator of these clusters as well as of the coding genes of T6SS-HIS-I related to chronic *P. aeruginosa* disease. In a process generally overlooked until now, RsmA has been demonstrated to act on most known T6SS genes, indicating that the type VI secretion system is also regulated by AmrZ (288).

The QS system in *Acinetobacter* spp. comprises the AbaR (receptor) and AbaI (synthase) proteins. These proteins are related to some virulence factors, such as motility, antibiotic resistance, survival properties, and biofilm establishment, in *Acinetobacter baumannii* and other *Acinetobacter* spp. (289, 290). In *Acinetobacter* strain M2 (originally named *A. baumannii* and later reclassified as *Acinetobacter nosocomialis*, due to genomic differences), synthesis of 3-hydroxy-C_{12}-HSL requires AbaI (221). More than one AHL has been found in 63% of *Acinetobacter* isolates. Nonetheless, there is no correlation between different AHLs and particular species of *Acinetobacter* (291). Deletion of the *abaI* gene, which is associated with AHL formation, reduced biofilm formation by 30 to 40% relative to that in the wild-type strain (290). However, addition of an exogenous AHL obtained from *Acinetobacter* restored biofilm formation in the mutant (292). Moreover, the important role of a new QS enzyme, AidA, in bacterial defense against 3-oxo-C_{12}-homoserine-lactone, an inhibitor of the quorum sensing system (293), was recently analyzed (294). Finally, in *A. baumannii* clinical strains from clone ST79/PFGE-HUI-1 (which lacks the AdeABC efflux pump) and a modified strain (named ATCC 17978 Δ*adeB*), the presence of bile salts (a stress condition) induced overexpression of genes involved in biofilm production, the T6SS, and surface motility, which are associated with QS (173).

One or more quorum sensing systems containing a synthase and an acyl-homoserine lactone receptor have been identified for all species of *Burkholderia* (295). Two complete quorum sensing systems, known as CciIR and CepIR, were discovered in *B. cenocepacia* J2315, in addition to a gene encoding a regulator known as CepR2 but lacking a synthase and, finally, a system based on *Burkholderia* diffusible signal factor (BDSF), known as RpfFBC (296–298). Biofilm formation in *B. cenocepacia* H111 is highly dependent on BapA, which is a surface protein, and BapR, a regulatory protein. Both the *bapA* and *bapR* genes need QS for high levels of expression (299). Moreover, in their study, Aguilar and collaborators reported that BapR is an important protein in relation to the development of persister cells, indicating that this regulator may be a useful target in the production of drugs to prevent the formation of biofilms and persister cells (299).

The complex *M. tuberculosis* biofilms can produce a subpopulation of drug-tolerant persister cells (300). In addition to persistence against antibiotics, biofilms can also be envisioned as being part of a key persistence strategy of *M. tuberculosis* against the host immune system in chronic infections, particularly those that do not display clinical symptoms (301). The QS system of *M. tuberculosis* is largely unknown, and we highlight expression of the WhiB3 protein in response to environmental signals present *in vivo*, which is consistent with a model of QS-mediated regulation (302).

(p)ppGpp Network

The (p)ppGpp response involves the enzymes guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp). Under starvation conditions (amino acid starvation) and other types of environmental pressure, the "alarmone" molecule is produced. The (p)ppGpp network includes Rel/SpoT homolog (RSH) proteins with a nucleotidyltransferase domain, some of which display only synthetic functions, only hydrolytic functions, or both (303, 304). Other proteins, such as the RelV (p)ppGpp synthase of *Vibrio* (305) and the RelQ (p)ppGpp synthase of Gram-positive bacteria, have an important role in the (p)ppGpp network (48). Cell processes such as replication, transcription, and translation are influenced by the (p)ppGpp network. Also, (p)ppGpp binds to RNA polymerase, which modifies the transcriptional profile and alters the

translational machinery (such as rRNA and tRNA) until nutritional conditions improve (306–308). Interestingly, bacteria deficient in (p)ppGpp production usually display massive defects in persister cell formation and survival (7).

Two different (p)ppGpp-dependent (RSH proteins and amino acid starvation conditions) (309) and -independent pathways have been studied in relation to salt tolerance in *E. coli* (310). In *E. coli*, the generation of the abnormal amino acid isoaspartate, which can be repaired by isoaspartyl protein carboxyl methyltransferase (PCM), is a specific type of protein damage that is significant for bacterial survival. The relationship between unrepaired isoaspartyl protein damage and the development of *E. coli* persisters through activation of the (p)ppGpp network was recently investigated (310, 311). Moreover, Harms and collaborators confirmed the important role of (p)ppGpp and Lon in the development of persister cells in *E. coli* (312).

Several pathways are involved in the (p)ppGpp network (RSH proteins) in *S.* Typhimurium (313), including (i) osmoregulation of periplasmic glucan content by expression of the *opgGH* operon (314); (ii) enhancement of the expression of stress-dependent genes incorporating genetic material acquired by horizontal transfer (315); (iii) regulation of virulence proteins required for intracellular survival in macrophages (316); (iv) aminoglycoside resistance (317); (v) a defense mechanism against reactive nitrogen species (RNS) encountered in the host through the RNA polymerase regulatory protein DksA (318); (vi) regulated expression of the virulence elements (motility and biofilm production) from pathogenicity island 1, which is necessary to facilitate processes such as invasion and intracellular replication in host cell lines, such as human epithelial cells, as well as uptake by macrophages (319–321); and (vii) development of persister cells by inhibition of bacterial growth interfering with FtsZ assembly (322).

Several functions have been associated with the (p)ppGpp network (amino acid starvation) in *V. cholerae*. In 2012, a study of *V. cholerae* investigated how biofilm formation is controlled by a tangled regulatory mechanism, with QS acting as a negative regulator, the restrictive response mediated by (p)ppGpp synthases (RelA, SpoT, and RelV) acting as a positive agent, and both interacting to coordinate the production of biofilm with the environmental conditions (323). Moreover, several studies have associated this system with the development of virulence elements, such as cholera toxin (CT) and toxin-coregulated pilus (TCP) (323–325). However, it has also been found that production of hemagglutinin (HA)/protease, which influences the formation of biofilm and motility in *V. cholerae*, is independent of the (p)ppGpp network but requires HapR, RpoS, and cyclic AMP receptor protein (CRP) (326). It was recently shown that (p)ppGpp positively controls the production of acetoin and that this specific role of (p)ppGpp in *V. cholerae* enables the pathogen to survive in environments with considerable concentrations of glucose, as in the human intestine (327). Finally, the (p)ppGpp network in this microorganism has been linked to RpoS, which manages the "mucosal escape response" by establishing a specific response and executing chemotaxis and motility through intracellular proteolysis (25, 328).

However, the extensive literature shows that different intracellular stress responses, such as the SOS response, ROS, and (p)ppGpp (RSH proteins), can transform a subset of *K. pneumoniae* cells into persister cells with antibiotic tolerance (Fig. 2) (329). In addition, ROS generation is induced by multiple factors, such as suboptimal concentrations of aminoglycosides (which also activate the SOS response), paraquat, and H_2O_2 (control by RpoS, SoxRS, and YjcC). It can thus be concluded that antimicrobial treatment can promote the persistence phenotype in *K. pneumoniae* (330).

H. pylori must withstand the inhospitable conditions of the human stomach and does so via a minimum number of transcriptional regulators which control the stringent response, as analyzed in different studies (331, 332). A *rel/spoT* homolog (RSH) gene-deleted mutant was incapable of surviving under extreme conditions of acidity and oxygenation, including during infection and transmission (332). Moreover, the Rel protein has been demonstrated to be vital for the persistence phenotype in *H. pylori* inside macrophages during phagocytosis in the gastric environment (333). In addition, CO_2 restriction considerably raises the level of (p)ppGpp and ATP inside this bacterium,

although it does not reduce the mRNA level, indicating activation of a stringent response (334).

In *C. jejuni* strains, the (p)ppGpp network (RSH proteins), together with the phosphohydrolases (PPX/GPPA) and polyphosphates [poly(P)], has been related to motility, biofilm production, and the ability to survive under stress conditions, such as nutrient deficiency, as well as in the process of invasion and the persistence phenotype within the host cell (335, 336).

In *E. faecalis*, the lack of (p)ppGpp production results in a lower ability to maintain biofilm development (337). In this pathogen, (p)ppGpp production is regulated by the Rel/SpoT (RSH) proteins RelA and RelQ synthetase (338). RSH activates a strict response so that changes in (p)ppGpp levels affect survival under stress conditions as well as the virulence capacity (339). In a vancomycin-resistant *Enterococcus faecium* (VRE) subpopulation, a mutation in the stringent response (SR) pathway was recently reported that caused an increase in reference (p)ppGpp levels, which triggered antibiotic tolerance inside the biofilm (340). Finally, alarmone levels regulate the responses against environmental stress, tolerance to antibiotic treatment, and virulence elements in *E. faecalis* (341, 342).

In *Shigella* spp., with the stringent response, the RSH proteins together with the DksA protein showed activity (343).

Little is know about the role of the (p)ppGpp network in bacteria such as *Yersinia enterocolitica* and *Clostridium difficile*.

Persistence phenotype infections produced by *S. aureus* are nutrient restriction reactions that directly depend on the (p)ppGpp mechanism (344). The involvement of (p)ppGpp signaling in the development of persister cells as well as in the acquisition of antibiotic tolerance by *S. aureus* cells has been described (344). Nevertheless, more detailed analysis is required in relation to molecular principles, as recent studies did not find any connection between (p)ppGpp signaling and the development of persister cells in *S. aureus* (345, 346). CodY24, a repressor, has been shown to regulate expression of *S. aureus* gene homologues of Rel/SpoT proteins (RSH proteins), i.e., *rsh* genes, which constitute a distinct class of (p)ppGpp synthase genes (347). Mutations in *codY* or in *rsh* were not found to have any consequences on the number of persister cells during growth (346). It has also been noted that *S. aureus* has putative GTPases that are capable of recognizing guanosine tetraphosphate and guanosine pentaphosphate with a high affinity (345). Further analysis confirmed that these are active GTPases regulated by the presence of ribosomes (activation) and (p)ppGpp (inhibition). Once these molecules were characterized, it became clear that bacteria have a mechanism whereby cell growth can be halted under stress conditions by activation of (p)ppGpp, which blocks the correct formation of 70S ribosomes (345).

In *P. aeruginosa* strains, the RelA/SpoT protein RSH (stringent response) and the RpoS protein (stress conditions) promote the tolerance of *P. aeruginosa* biofilms to ciprofloxacin but not to tobramycin (Fig. 2) (348). RSH proteins have been described for this pathogen (349). Interestingly, in *Acinetobacter oliveivorans* DR1, QS controls (p)ppGpp synthase (RSH proteins), AHL, and histidine kinase proteins during participation in biofilm production and hexadecane metabolism (350).

As for *cis*-2-dodecenoic acid factor (discovered in *B. cenocepacia*), it belongs to the diffusible signal family and has been associated with increased levels of RecA (SOS response) and (p)ppGpp (RSH proteins) and a reduction in biofilm formation (Fig. 2) (351).

M. tuberculosis contains homologues of Rel proteins that react to low nutrient levels via activation of (p)ppGpp (352, 353). These proteins are necessary for growth in aerobic and anaerobic environments as well as for long-term survival in response to starvation (352), and they induce drug tolerance (354). In guinea pigs, cells which had lost Rel proteins were associated with a remarkable lack of tubercle lesions as well as an absence of caseous granulomas in histological sections (355). Interestingly, the success of the *M. tuberculosis* pathogen depends on cell growth in the host, regulated by (p)ppGpp (RSH proteins) and CarD (356). The lack of the CarD protein led to killing of

M. tuberculosis due to DNA damage, starvation, and oxidative stress, all of which led to a reduction of rRNA transcription (Fig. 2) (356).

Toxin-Antitoxin Systems

Finally, one of the best-studied mechanisms of formation of persister cells involves toxin-antitoxin (TA) systems, which trigger a state of bacterial dormancy to evade the effects of drugs or stress conditions (8). TA systems are small genetic systems located on bacterial plasmids as well as on chromosomes. TA loci usually are comprised of two genes, which encode a stable toxin and an unstable antitoxin that inhibits the toxin. TAs are currently divided into six distinct classes on the basis of the proteomic nature of the corresponding antitoxin (1, 16). See Fig. 4 for an explanation of the six best-studied types of TA modules. Critically, deletion of a single TA system that reduces persistence under certain conditions has been shown for the *mqsR/mqsA* locus (12, 357), the *tisB/istR* locus (181), and the *dinJ/yafQ* locus (358). Finally, several TA systems are triggered by the SOS system and (p)ppGpp to drive the development of persister cells (Fig. 2) (181, 359).

Research on the TA modules of *Escherichia coli* has provided most of the information that exists regarding persister formation (360). Also, type I, type II, and type IV TA loci are localized in the cryptic prophages of *E. coli* (361). In 2011, a model of *E. coli* persister formation based on the expression of 10 mRNAs for type II TA endonuclease modules was proposed (362). However, we must point out that that study was recently retracted due to the discovery of inadvertent artifacts (312). The following class II TA modules have been described: (i) the HipBA TA module, (ii) the TisB/IstR TA module, (iii) the HokB/SokB TA module, (iv) the YafQ/DinJ TA module, (v) the MazEF TA module, and (vi) the MqsRA TA module. The *hipA* toxin gene was the first gene to be associated with the production of persister cells (50, 363). TA loci encoding mRNases and persistence development are closely related (364, 365). In addition, an increment of the expression of HipA toxin in *E. coli* has been reported to halt growth by boosting (p)ppGpp production via RelA, a signal commonly related to amino acid requirements (365). By use of chloramphenicol to inhibit (p)ppGpp production in HipA-arrested cells, effects such as halting the initiation of replication and RNA synthesis were reduced, thus enabling recovery of susceptibility to β-lactam antibiotics (365). The TisB/IstR TA module is activated by the SOS system. TisB works as an ion channel which reduces proton motive force and the amount of ATP, enhancing the formation of persistent cells and causing antimicrobial tolerance (180). The HokB/SokB TA module is regulated by the (p)ppGpp system (181, 359). For the YafQ/DinJ TA module, the toxin shows an association with increasing persistence by reducing indole, thus establishing a relationship between the TA systems and the cell signaling QS system (224). The MazF toxin, which generally breaks down cellular RNAs, halts cell growth for some time and promotes formation of persister cells (366). The MqsR toxin influences persister cell formation (357). Moreover, the MqsRA TA in *E. coli* is physiologically vital for survival of bacterial cells in the gallbladder and upper intestinal tract, where concentrations of bile are generally high (367). As previously mentioned, TA systems promote persister states in bacteria by repressing bacterial metabolism or inhibiting cellular growth by the toxin. This state leads to bacterial tolerance of environmental stressors (368).

The presence of *Salmonella* inside macrophages creates stress conditions for the bacterium and induces the production of a subpopulation of persister bacteria by class II TA modules (369). Fourteen putative class II TA modules have been described for this pathogen, all of which are related to the development of persister cells inside macrophages. Among these, the toxin (TacT) contributes to the formation of these persisters in *Salmonella* populations by transiently blocking translation (370). The TacT toxin is an acetyltransferase that inhibits translation of tRNA molecules by disrupting the primary amine group of the amino acids, also favoring the formation of persister cells by the pathogen (370).

For *V. cholerae*, TA loci have been described as being present in a superintegron. *V. cholerae* TAs were assigned to the Phd/Doc, HigBA, RelBE, and HigBA families (371).

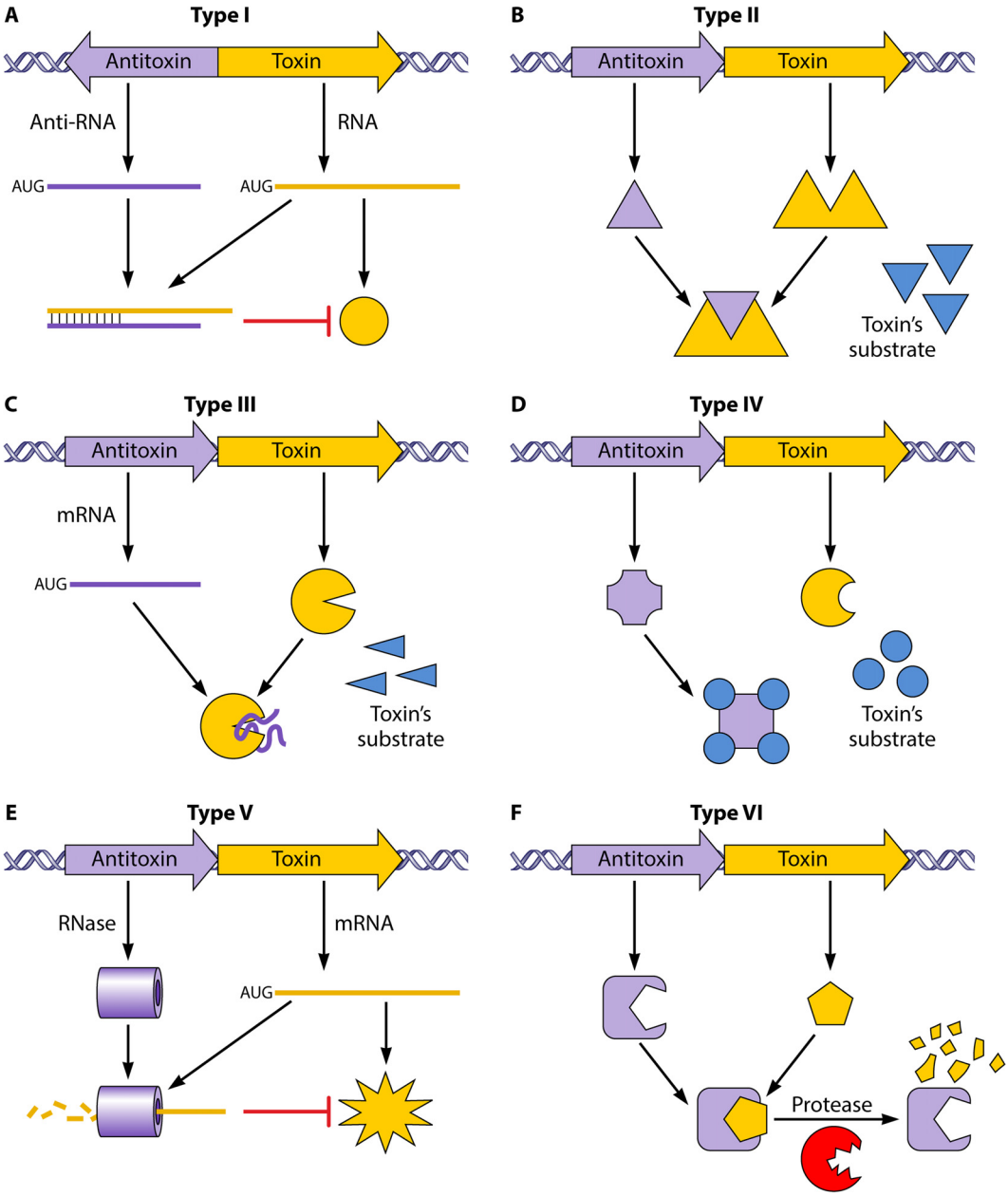

FIG 4 Graphical representations of antitoxin-toxin interactions and their classification in the different types of TA systems in clinical pathogens. (A) Type I. There is an interaction between mRNAs that blocks toxin protein formation. (B) Type II. The antitoxin protein forms a complex with the toxin protein, inhibiting its function. (C) Type III. The antitoxin mRNA generates a complex with the toxin protein, inhibiting its function. (D) Type IV. The antitoxin protein competes with the toxin for its substrates. (E) Type V. The toxin mRNA is degraded by an RNase encoded by the antitoxin gene. (F) Type VI. A TA complex is formed by the union of toxin and antitoxin proteins, producing decomposition of the toxin by a cellular protease. (Adapted from reference 16.)

However, only RelBE-family TA systems in *V. cholerae* are involved in biofilm and ROS generation (Fig. 2) (371).

Klebsiella pneumoniae isolates have a major role in antibiotic resistance, and the genes for several type I and II TA systems (Hok/Sok, PemK/PemI, and CcdA/CccB) (16) have previously been identified on resistance plasmids carried by this bacterium (372). A bioinformatics approach was used to analyze the distribution of the locus of the type II system and to determine the variability in the TA loci from 10 complete sequenced genomes of *K. pneumoniae*, revealing numerous putative type II TA loci (373). Some RelBE-like TA systems were also distributed in a manner different from that

for the *K. pneumoniae* RelBE systems (59). Thus, the distributions of RelBE_1kp and RelBE_2kp loci differ in plasmids and chromosomes, although they were found in the same *K. pneumoniae* isolate (16, 373). However, a detailed distribution and the implications for the development of a persistence phenotype in *K. pneumoniae* are unknown (373).

A new type I TA system, the AapA1/IsoA1 locus, present on the chromosome of *H. pylori*, was recently characterized (374). Several type II TA systems associated with the bacterial persistence phenotype have also been localized in this pathogen, including (i) the HP0894-HP0895 proteins (375, 376), (ii) the HP0892-HP0893 proteins (RelE-family TA system), and (iii) the HP0967-HP0968 proteins (Vap family) (374).

CjrA/CjpT (type I) and VirA/VirT (type II) TA systems were identified in *Campylobacter* spp. (377). These systems were encoded by the pVir plasmid, which is involved in virulence and natural transformation. VirT belongs to the RelE family, one of the best-studied TA systems (374, 377).

Several plasmids encode toxin-antitoxin systems in strains of *Enterococcus* spp., as follows: (i) the plasmid pAD1-encoded Fst toxin (type I toxin-antitoxin) in *E. faecalis* affects the permeability of the membrane, thus altering the cellular responses to antibiotics (378–382); and (ii) plasmids encoding vancomycin resistance and harboring genes for the ω-ε-ζ Par toxin-antitoxin and Axe-Txe toxin-antitoxin have been located, but their involvement in the presence of persister cells has not been analyzed (383–386). Moreover, *mazEF*, *mazEG*, and *higBA* loci have often been found in *E. faecalis* and *Enterococcus faecium* clinical strains (387).

On the other hand, type II TA systems are related to bacterial persistence in *Shigella* sp. isolates and include YeeUT (388, 389), VapBC (390, 391), GmvAT, and CcdAB (392), while the only system described for *Yersinia enterocolitica* is the CcdA/B TA system, and its function has not been analyzed (393).

Only one type II TA system has been described for *C. difficile*, namely, MazEF (endoribonuclease), which is involved in *C. difficile* sporulation (68). However, by means of the RASTA-Bacteria TA database, additional putative TA systems, the COG2856-Xre and Fic systems, are assumed to exist in strain 630 of *C. difficile* (394).

S. aureus harbors some annotated chromosomal TA systems, and their function in persister formation is of interest (395). Several type II toxin-antitoxin systems have been identified in *S. aureus*, including MazEF and Axe1/Twe1 as well as Axe2/Twe2, both of which are RelBE homologues. A recent study showed that the two open reading frames directly over the *sigB* operon region in the *S. aureus* genome, herein designated *masES* and *mazFS*, represent a TA system (396). However, further studies of the involvement of these TA systems in the development of persister cells must be performed. Although the findings of bioinformatics studies of *P. aeruginosa* suggest that TA systems are abundant in the genome of this bacterium (397), the functions of these systems have not been established (16), except for the HigBA system, which downregulates virulence by the effect of the HigB toxin, which reduces the production of pyocyanin and pyochelin and surface motility (398).

The plasmid p3ABAYE is the most frequently found plasmid among the toxin-antitoxin plasmidic systems in *A. baumannii*. This plasmid (94 kb) probably encodes the following five TA systems: (i) RelBE; (ii) two HigBA systems, encoded in opposing directions; (iii) SplTA (DUF497/COG3514 domain proteins); and (iv) CheTA (HTH/GNAT domain proteins). These were found in a group of *A. baumannii* clinical strains from Lithuanian hospitals. The HigBA and SplTA TA systems were particularly common (88.6% predominance, taking into account the 476 clinical samples). Remarkably, in 46 of the *A. baumannii* isolates analyzed, expression of the HigBA toxin-antitoxin system was not observed (399, 400). All of these toxin-antitoxin systems were found in most clinical isolates of *A. baumannii* belonging to the ECI and ECII groups worldwide. The function of SplTA was found to be associated with the persistence phenotype (399). Moreover, the AbkB/AbkA toxin-antitoxin system, also known as SplTA, was found to be encoded by the most frequent resistance plasmid of *A. baumannii*, which carries an OXA24/40 β-lactamase (carbapenem resistance) gene (401). The toxin of this system

has the ability to prevent translation when overexpressed in *E. coli*, through the scission of *lpp* mRNA plus the transfer of mRNA, all of which indicate that the AbkB toxin acts as an endoribonuclease. The stability of the plasmid in the absence of selection pressure can be explained by the presence of the AbkB/AbkA system, especially in the case of small plasmids pAC30a and pAC29a, which lack a bla_{OXA24}/bla_{OXA40}-like gene (401). Moreover, in a recent study of isolates of *A. baumannii* with resistance to carbapenem due to the presence of a plasmid encoding OXA24 β-lactamase and the AbkAB TA module, we analyzed the presence of a link between molecular mechanisms associated with the development of tolerance to a biocidal compound (chlorhexidine) and later formation of a subgroup of persister cells in the presence of an antibiotic (imipenem). These persister cells showed overexpression of the *abkB* toxin gene as well as downregulated expression of the *abkA* antitoxin gene (349).

Expression of most of the toxin genes (from TA systems) in biofilm cells of *B. cenocepacia* is upregulated relative to that in planktonic cells (82, 402). The high levels of expression of these bacterial toxins involve an increase in cellular survival after treatment with ciprofloxacin or tobramycin, confirming the importance of the toxins in the development of persister cells as well as the generation of antibiotic tolerance in biofilms (82, 402). The FixL toxin was recently identified in *Burkholderia dolosa*, which belongs to the *B. cepacia* complex (BCC). This toxin is homologous to FixL, which forms part of the two-component FixL/FixJ system of the *Rhizobiales*. In *B. dolosa*, the *fixL* gene acts as a general regulator of motility, biofilm formation, persistence, virulence, and intracellular invasion (45).

Finally, the genome of *M. tuberculosis*, a particular persistence phenotype pathogen, has an abundance of toxin-antitoxin systems. Most of these modules have been found to be functional *in vivo* in animal models, and they are involved in virulence and antibiotic tolerance (403–406). To date, numerous toxin-antitoxin systems (confirmed and putative) have been identified in the H37Rv isolate of *M. tuberculosis*; the most frequent are type II TAs (VapBC, MazEF, YefM/YoeB, RelBE, HigBA, and ParDE) (Fig. 5) (404). Interestingly, no other type of TA system has been detected in this bacterium (407, 408). Most *M. tuberculosis* systems (409) have been analyzed empirically, in *Mycobacterium smegmatis* and *E. coli*, with the aim of determining the toxin function in growth restriction as well as how to neutralize this function. Thus, researchers found that 37 of the TAs were functional under at least one condition (404, 405, 410–413). The articles cited summarize the current knowledge of the *M. tuberculosis* toxin-antitoxin modules. Moreover, analysis of the *M. tuberculosis* persister transcriptome shows positive regulation of the toxin-antitoxin systems (213, 414). Although the involvement of different TA modules in this repertoire is not fully understood, functional specialization can be considered due to the synergy observed under certain stress conditions (405, 406).

We summarize the molecular mechanisms of tolerance and persistence previously described for each pathogen in Table 1.

MEASUREMENT OF LEVELS OF BACTERIAL TOLERANCE AND PERSISTENCE

Survival of bacteria in natural environments depends directly on the capacity of the microorganisms to sense and accommodate to the surroundings. Bacteria can recognize and modify themselves in different situations by monitoring the environment and also by creating signals and modifying gene expression accordingly.

In a recently published opinion article, Brauner and collaborators highlighted the importance of distinguishing between resistant, tolerant, and persistent bacterial cells before selecting the appropriate antibiotic treatment (5). With the aim of establishing differences in the diverse survival strategies under stress conditions, they propose measurement of the minimum duration of killing (MDK) in batch cultures of bacteria. The MDK, which is based on the concept of effective killing, is used to provide a quantitative measure of tolerance and indicates that tolerant strains must be exposed to the condition under consideration for a longer time than that for susceptible strains. The MDK is described as the usual time required by an antimicrobial to eliminate a

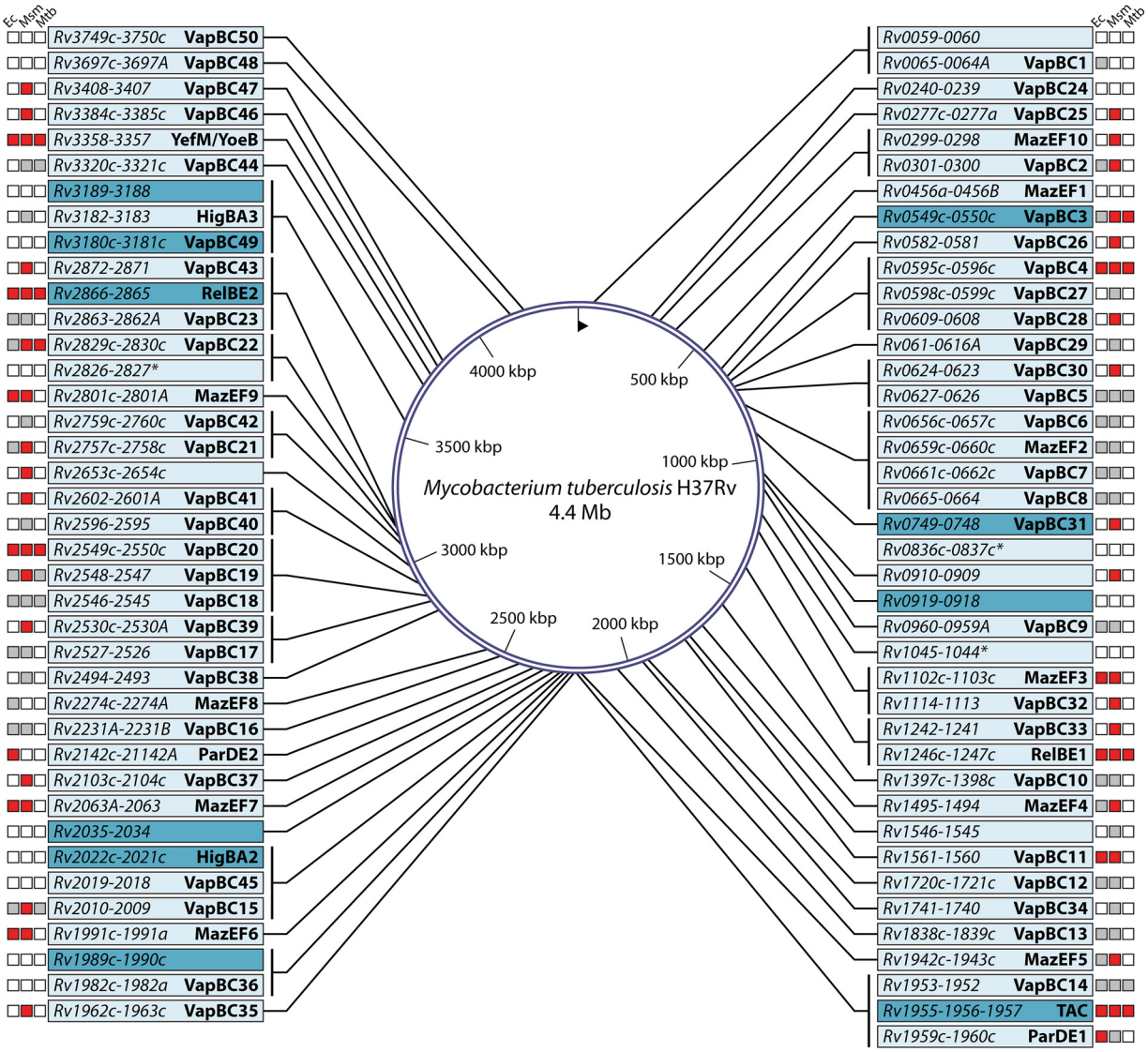

FIG 5 Chromosomal map of *M. tuberculosis* H37Rv TA systems. TA systems are annotated according to the information in the Tuberculist database, except for VapBC45 (Rv2018-Rv2019), VapBC49 (Rv3181c-Rv3180c), VapBC50 (Rv3750c-Rv3749c), HigBA2 (Rv2022c-Rv2021c), HigBA3 (Rv3182-Rv3183), YefM/YoeB (Rv3357-Rv3358), and MazEF10 (Rv0298-Rv0299). Most of the TA systems depicted here probably belong to type II, except for those marked with an asterisk, which are putative type IV systems. For each system, the functionality in *E. coli* (Ec), *M. smegmatis* (Msm), and *M. tuberculosis* (Mtb) is depicted as follows: red indicates inhibition of growth, gray indicates no inhibition of growth, and white indicates that the system was not tested. The 10 most commonly induced TA systems in drug-tolerant persister cells are highlighted by a dark blue background. (Reproduced from reference 404 with permission.)

certain proportion of the bacterial culture (5, 415). The MICs of antibiotics are similar for tolerant and susceptible isolates. However, the MDK_{99} (time to kill 99% of the cells in the culture) is generally higher for tolerant than for susceptible isolates. Furthermore, the MIC and MDK_{99} for persister cells in bacterial populations are also similar to those observed for susceptible bacteria; however, for cells in culture, the $MDK_{99.99}$ is considerably higher for persister cells than for susceptible cells (Fig. 5) (5). A recently described novel method, the TDtest, would be capable of detecting distinct levels of antibiotic tolerance in clinical isolates. This technique was analyzed with *E. coli* isolates, which were also used to study the antimicrobials with the highest activity against tolerant populations as well as persister subpopulations (Fig. 6) (416, 417).

NEW APPROACHES TO TREATMENT OF BACTERIAL PERSISTENCE

The development of new bacterial treatments must include tolerant and persister bacterial cells as targets. Hence, knowledge of the mechanisms of bacterial tolerance or

TABLE 1 Targets of different mechanisms associated with bacterial persistence[a]

Environment and pathogen	Target(s)							
	Stress response	ROS response	Energy metabolism	Efflux pump	SOS response	Quorum sensing (QS)	(p)ppGpp signaling	Toxin-antitoxin (TA) system
Gastrointestinal tract								
E. coli	RpoS (21, 22)	Superoxide dismutase (SOD)/catalase (50)	Cytochrome *bd* oxidases (95, 98), cytochrome *c* peroxidases (101), TauD protein (132, 133)	AcrAB (147, 152)	RecA/RecBCD (178, 179), TisB toxin (180)	SdiA/LuxS (AI-2/AI-3 autoinducers/indole) (221), OxyR/phage shock (222, 223)	RSH proteins (309), PCM[b] (310, 311)	HipBA (363–365), TisB-IstR (179), HokB-SokB (181, 359), YafQ/DinJ (224), MazEF (366), MqsRA (357, 367)
Salmonella spp.	RpoS (23)	SoxRS, OxyR, σ^S and σ^E factors, SlyA protein, *dps* gene (51)	Cytochrome *bd* oxidases (98)	AcrAB (11, 154), AcrD (57), MdtD (88), MacAB (154)	RecA (176)	Unknown synthase/SdiA (3OC8HSL signal) (230); LuxS, LsrB (AI-2 signal) (231); QseB, QseC (AI-3 signal) (232); T3SS/T6SS (234)	RSH proteins (313), *opgGH* operon/stress-dependent genes/DksA/FtsZ interference (314–319, 322)	Putative class II TA systems/TacT (370)
Vibrio spp.	RpoS (25)	Catalases (KatB-KatG)/PhoB-PhoR system (53), OxyR (54), cholix (55)	Oxygen/reductases (102–105), taurine (136–139)	EmrD-3 (156)	RecA (185)	LuxS, LuxP (AI-2 signal)/CqsA, CqsS (CAI-1 signal)/AlsR/AphA (235–238); T3SS/T6SS (241–245)	RSH proteins (323), cholera toxin (CT)/toxin-coregulated pilus (TCP)/acetoin (324–327)	Phd-Doc/RelBE/HigBA/ParDE (371)
Klebsiella spp.	RpoS/SoxRS (26)	CPS (58)		AcrAB/OqxAB (158)	RecA/Viz/GrapE/ClpX/Dead proteins (186)	LuxS (AI-2 signal) (246, 247), T6SS (249)	RSH proteins (329), SoxRS/YjcC (330)	Hok-Sok/PemK-PemI/CcdA-CccB/RelBE (372, 373)
Helicobacter spp.	Fur/HspR (31)	Catalase/SOD/arginase (62, 63)	Cytochrome *bd* oxidases (109–111), taurine (140)	Two efflux pumps (159)	RecA/AddAb (187, 188)	LuxS (AI-2 signal) (250–252)	RSH proteins (332, 333)	AapA1-IsoA1/RelE family (HP0894-HP0895)/Vap family (HP0967-HP0968) (374–376)
C. jejuni		KatA-SodB/AhpC-Tpx/Bcp (64)	Cytochrome *bd* oxidases (112)	CmeABC (151, 160)	RecA (189)	LuxS (AI-2 signal) (160), T6SS (151, 261)	RSH proteins/PPX/GPPA/PolP (335, 336)	CjrA-CjpT/VirA-VirT (374, 377)
Enterococcus spp.	Gsp65 (*ohr* gene) (32), Gsp62 (33), Gls24 (34–36)		Cytochrome *bd* oxidases (113), Clp ATP protease (114)	MefA/TetK/TetL/MsrC (161)	RecA/UmuDC (190)	*fsrA/fsrC/gelE*/GTF genes (262)	RSH proteins (338)	Fst toxin (pAD1) (378–382), ω-ε-ζ (pw9-2)/Axe-Txe modules (pRUM) (383–386), MazEF/MazEG/HigBA (387)
Shigella spp.		Toxin (65)	Cytochrome *bd* oxidases/CydC (115, 116)	AcrAB (162), MdtJI (163)	RecA/topoisomerases/histones (191)	AI-2 (signal) (263), T3SS/T6SS (264–266)	RSH proteins/DksA (343)	YeeUT/VapBC/GmvAT/CcdAB (388–392)
Yersinia spp.		New SODs (66), yersiniabactin (67)		RosA/RosB (164)	RecA/histone-like proteins (192)	LuxI/LuxR-like proteins (267)		CcdAB (393)
C. difficile	HSP proteins (GroESL/DnaKJ) (40)	Catalase/SOD (68), TcdA-TcdB/glutamate dehydrogenase (GDH) (69)		CdeA (165)	RecA/LexA (193, 194)	LuxS/SpoOA (271, 272)		MazEF (68), COG2856-Xre/Fic (394)

(Continued on next page)

TABLE 1 (Continued)

Environment and pathogen	Target(s)							
	Stress response	ROS response	Energy metabolism	Efflux pump	SOS response	Quorum sensing (QS)	(p)ppGpp signaling	Toxin-antitoxin (TA) system
Respiratory tract								
S. aureus	σ^B factor (42)	Catalase/SOD (70), aconitase/succinate dehydrogenase-TCA cycle enzymes (71)	Cytochrome *bd* oxidases (117), CymR (taurine) (118, 142)	Qac efflux (166)	RecA/LexA, RexAB/PolV (195, 196)	SarA/Agr (AI-2 signal) (273–276), LuxS/IcaR (275)	RSH proteins, *rsh* genes (344–346), CodY24 (347), GTPases (345)	MazEF/RelBE (395, 396), TisB-GhoT toxins (346)
P. aeruginosa	RpoS/*pls* locus (41)	Catalase/SOD/POX (74), mucoid phenotype (75, 76)	cbb_3-type enzyme (118), GacAS system (123, 124)	MexAB (167, 168, 172)	RecA/LexA/PrtR/PA0906 (198, 201, 203), UmuDpR (205)	Las/Rhl QS systems (AI-2/PQS, AIP/AHL/DSF signals) (277, 278, 283), T6SS (RsmA/AmrZ) (286–288)	RSH proteins (348)	HigBA (398)
A. baumannii		SOD (79), catalases (KatA/KatE/KatG/KatX) (80)	Cytochrome *bd* oxidases (128), *tauRXYPI* operon (143)	AdeABC, AdeFGH (170–173)	RecA/LexA, UmuC (207–209)	AbaR/AbaI/AidA (*N*-[3-OH-C_{12}] signal) (289–291, 294), T6SS system (173)	RSH proteins (349)	RelBE/HigBA/CheTA/AbkAB (SpltA) (349, 399–401)
B. cepacia	RpoN/RpoE (*fixLJ*) (43–45)	SOD/catalase, glyoxylate (TCA cycle) (83)	*tauABC* operon (145)	BCAM0925 to -0927, BCAM1945 to -1947, BCAL1672 to -1676 (RND efflux) (174)		CepIR/CciIR/CepR2/RpfF (BapA and BapR) (296–298)	RSH proteins/DSF signals (351)	FixLJ (45)
M. tuberculosis		Catalase (KatG) (84)	*pst* operon (129–131)		ImuA-ImuB/DnaE2 (212), ClpR-like protein (215)	WhiB3 regulator protein (302)	RSH proteins/CarD protein (352, 353)	VapBC/MazEF/YefM/YoeB/RelBE/HigBA/ParDE (193, 403–415, 480)

[a]Numbers in parentheses are reference numbers.

[b]Isoaspartyl protein carboxyl methyltransferase.

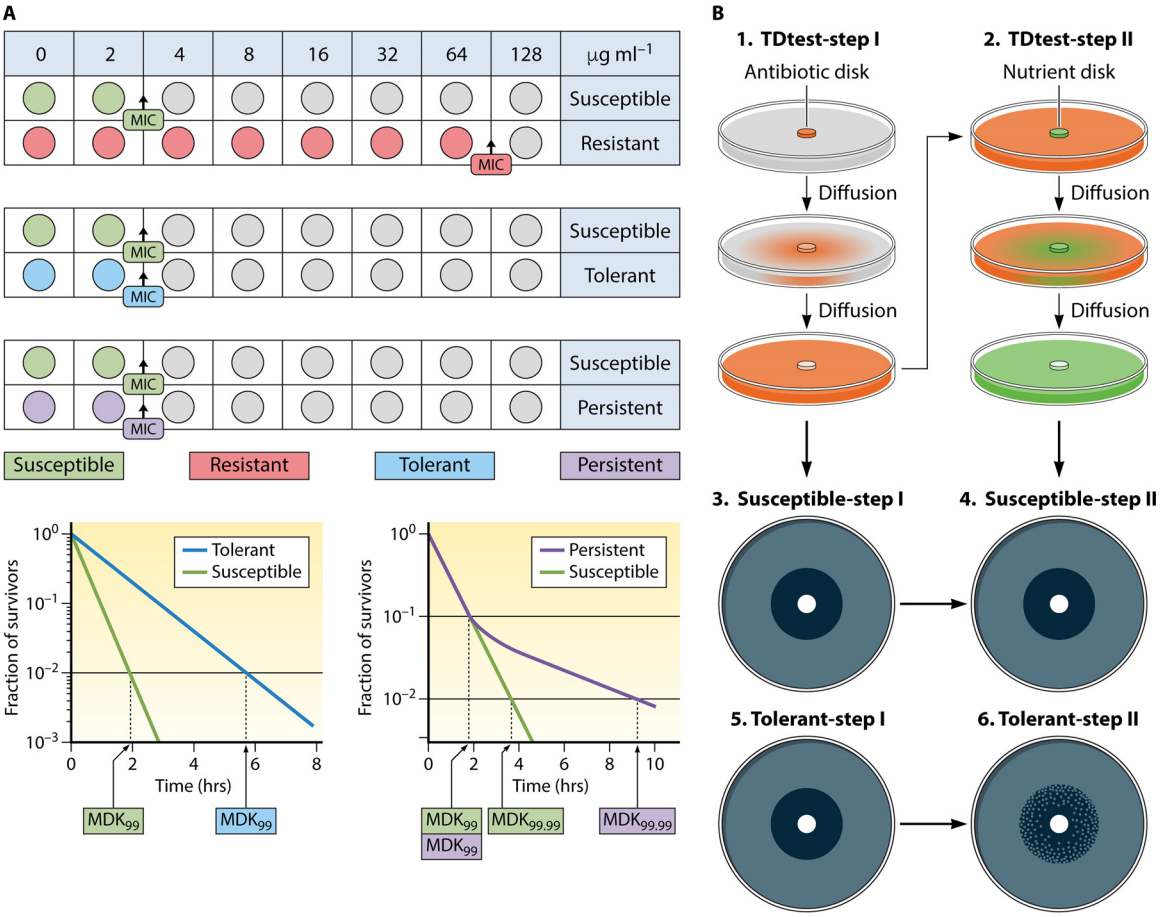

FIG 6 (A) Characteristic drug responses for resistance, tolerance, and persistence phenotypes. The MIC value is useful for analyzing bacterial populations that are susceptible and resistant to antibiotics, while the MDK (minimal duration of bacterial killing) is a quantitative measure that is suitable for studying tolerant and persistent bacterial cells. Among susceptible bacterial cells, the MDK_{99} (time to kill 99% of culture cells) is higher for tolerant bacterial cells, while the $MDK_{99,99}$ (time to kill 99.99% of culture cells) is higher for persister bacterial cells. (Adapted from reference 5 with permission from Springer Nature.) (B) Analysis of bacterial tolerance and persistence in clinical strains by use of a modified disk diffusion test (TDtest). (1) A disk with drug is added to the agar plate. (2) The drug-infused disk is replaced by a glucose-infused disk. The drug diffuses from the disk. (3 and 4) Susceptible bacteria show growth inhibition around the drug-infused disk. (5 and 6) Tolerant bacteria show growth inhibition around the drug-infused disk (5), and colonies inside the inhibition zone after addition of glucose show late-growing bacteria of this isolate (6). (Adapted from reference 416.)

persistence may provide targets for development of new anti-infective treatments for combating tolerant and persister cells (61), including antimicrobial peptides (synthetic and pyocins/bacteriocins), antivirulence compounds, phage therapy, anticancer drugs, and new molecules (Table 2).

Use of a peptide prevented biofilm development and also yielded removal of mature biofilms of Gram-positive and Gram-negative pathogens, such as *A. baumannii*, *P. aeruginosa*, *E. coli*, methicillin-resistant *S. aureus*, *K. pneumoniae*, *B. cenocepacia*, and *S.* Typhimurium (418). Interestingly, the activity of inhibitory compounds analogous to Rel proteins [synthetic (p)ppGpp] has been described for *Mycobacterium* spp. (419). The impacts of these molecules on long-term persistence, biofilm disruption, and down-regulation of (p)ppGpp have been analyzed *in vivo* in *M. smegmatis* (419). Another compound, pyrazinoic acid (POA), has been described for *M. tuberculosis* and showed significant inhibition of persister cells (420). Finally, we highlight the peptide SAAP-148, which has shown an important level of activity against persister cells of methicillin-resistant *S. aureus* and MDR *A. baumannii* (421). In contrast, *P. aeruginosa* protects itself from other strains of its species by use of the R-type pyocin (422), which is structurally similar to the contractile tail of the *Myoviridae* bacteriophage family (423) and is encoded by a unique cluster in the genome; although the pyocin mainly kills *P.*

TABLE 2 Experimental treatments against persister cells

Treatment type	Molecule(s)	Mechanism of action	Bacterial target(s)	Reference(s)
Antimicrobial peptides	Peptide 1018	Blocking (p)ppGpp	Gram-negative pathogens: *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, *S.* Typhimurium, *B. cenocepacia* Gram-positive pathogens: methicillin-resistant *S. aureus*	418
	Peptide analogue RelA proteins	Blocking (p)ppGpp Antibiofilm activity	*M. smegmatis*	419
	Pyrazinoic acid (POA)	Blocking (p)ppGpp	*M. tuberculosis*	420
	Peptide SAAP-148	Biofilm inhibition	*S. aureus*, *A. baumannii*	421
	R-type pyocins	Bacterial lysis	*P. aeruginosa*, *Haemophilus* spp., *Neisseria* spp., *Campylobacter* spp.	423–434
	Curvacin A (bacteriocin)	Bacterial lysis	*L. monocytogenes*	439
	Enterocidin B3A-B3B (bacteriocin)	Bacterial lysis	*L. monocytogenes*	440
	Enterocidin B3A-B3B/nisin (bacteriocin)	Bacterial lysis	*L. monocytogenes*	440
Antivirulence compounds (QS inhibitor molecules)	QS inhibitory molecules	Inhibition of the MvfR virulence regulon	*P. aeruginosa*	441
	Halogenated indoles	LuxR inhibition	Gram-negative pathogen: *E. coli* Gram-positive pathogen: *S. aureus*	226
	(*Z*)-4-Bromo-5-(bromomethylene)-3-methylfuran-2-(5H)-one	Quorum sensing inhibition	*E. coli*	442
	Benzimidazole derivative M64	Blocking PqsR	*P. aeruginosa*	441
	RNAIII-inhibiting peptide (RIP) and its analogues	Inhibition of phosphorylation of target of RNAIII-activating protein (TRAP)	*S. aureus*	447–449
	Synthetic autoinducer of AIP and analogues (I to IV)	Inhibition of RNAIII	*S. aureus*	447
	Nonfunctional AIP analogues	Repression of many AgrC receptors (I to IV)	*S. aureus*	450
	Halogenated compounds	Quorum sensing inhibition	*P. aeruginosa*	221, 451
	AHL antagonists		*P. aeruginosa*	
	Cell extracts and secretion products		*P. aeruginosa*	
	QS inhibitors from food and plant sources		*P. aeruginosa*	
	Acylases and lactonases		*P. aeruginosa*	
	OmpA inhibitor	Inhibition of pathogenesis	*A. baumannii*, *P. aeruginosa*, *E. coli*	453
Phage therapy	Phage cocktails	Antibiofilm activity	Coagulase-negative staphylococci, *S. aureus*, *E. faecalis*, *E. faecium*, *E. coli*, *P. mirabilis*, *K. pneumoniae*, *P. aeruginosa*, *Acinetobacter* spp.	454–459
	Lytic phage phiIPLA-RODI		*S. aureus*	460
	Endolysins	Antibacterial effect	*S. aureus*	461
			Gram-positive bacteria other than *S. aureus*	462

(Continued on next page)

TABLE 2 (Continued)

Treatment type	Molecule(s)	Mechanism of action	Bacterial target(s)	Reference(s)
	PlyE146 endolysin	Antibacterial effect	*A. baumannii*, *P. aeruginosa*, *E. coli*	463
	LysAB2 endolysin	Antibacterial effect	Methicillin-resistant *S. aureus*, *A. baumannii*, *E. coli*, and other bacteria (with modifications)	464, 465
	CF-301 lysin	Biofilm agent	*Staphylococcus* spp.	481
Anticancer drugs	5-Fluorouracil, gallium compounds, mitomycin C, cisplatin	Inhibition of persister cells	*P. aeruginosa*	466–468
New molecules	DG70 (biphenyl benzamide)	Inhibition of respiration	*M. tuberculosis*	469
	Suramin	Inhibitor of RecA protein and SOS response	*M. tuberculosis*	471
	3-(4-[4-Methoxyphenyl] piperazin-1-yl) piperidin-4-yl biphenyl-4-carboxylate	Waking of persister cells by unknown mechanism	*E. coli* *P. aeruginosa*	472
	Antibiotic acyldepsipeptide ADEP4	ClpP protease activation	*S. aureus*	474
	Sytox Green NH125 (1-hexadecyl-2-methyl-3-[phenylmethyl]-1H-imidazolium iodide)	Permeabilization of the membrane	*S. aureus*	475
	Pyrazinamide (analogue of nicotinamide)	*trans*-Translation inhibition	*M. tuberculosis*, *B. burgdorferi*	476
	Daptomycin	Disruption of multiple aspects of bacterial cell membrane function	*B. burgdorferi*	417
	cis-2-Decenoic acid	Antibiofilm activity	*P. aeruginosa*	478
	Itaconate plus tobramycin	Inhibitor of ICL (isocitrate lyase)	*B. cepacia*	83
	Morin, pyrrolidine, quercetin, quinine, reserpine	Antibiofilm activity	*S. aureus*	479
	Small molecules	LexA autoproteolysis		203

aeruginosa, it can also kill members of other genera, such as *Neisseria*, *Campylobacter*, and *Haemophilus* (424–429). Bacteriocins, such as pyocins, have been reported for other Gram-positive and Gram-negative bacteria (430–434). Bacteriocins are a diverse group of ribosomally produced antimicrobial peptides. Some bacteriocins undergo extensive posttranslational modifications, which, together with their mode of action, have been used for classification purposes (435). Moreover, bacteriocins are toxic bacterial peptides that are released in order to inhibit bacterial growth and biofilm production (18, 436, 437). The most recent classification scheme (438) suggests three classes based on the mechanisms of biosynthesis and biological activity of lactic acid bacterium (LAB) bacteriocins, although it may also be applied to bacteriocins of other microorganisms. The use of bacteriocins in antimicrobial packaging is particularly well suited for foods at risk of surface contamination (435). We highlight two studies of the use of bacteriocins as an antibiofilm strategy. In the first of these, researchers used curvacin A-producing *Lactobacillus sakei* CRL1862 to reduce the biofilm formation of *Listeria monocytogenes* (439). In the second study, Al-Seraih and collaborators analyzed the capacity of enterocidin B3A-B3B alone and in combination with nisin (another bacteriocin) to inhibit biofilm formation in *Listeria monocytogenes* (440).

Antivirulence treatments aim to inhibit bacterial virulence without affecting growth of the bacteria (150). In 2014, in a study analyzing persister cell strains of *P. aeruginosa*, QS molecules that inhibited the MvfR virulence regulon (LysR-type transcriptional regulator) limited lethal effects in mice (441). Moreover, halogenated indoles have been observed to remove bacterial biofilms and persistent cells formed by Gram-positive and

Gram-negative microorganisms, such as *S. aureus* and *E. coli* (226). Pan et al. demonstrated that the QS inhibitor BF8 decreases the persistence of growing *E. coli* cultures and throwback antibiotic tolerance in the persisters (442). The benzimidazole derivative M64 is another QS inhibitor that has been described in relation to preventing the development of persister cells, by blocking the PqsR QS/virulence system of *P. aeruginosa* (441). However, resistance to these compounds is common (443–445) and increasing (446). Other inhibitory QS *S. aureus* peptides have also been evaluated and include the following. (i) The RNAIII-inhibiting peptide (RIP) and its analogues, which inhibit phosphorylation of a target protein (target of RNAIII-activating protein [TRAP]), leading to suppression of virulence characteristics *in vitro* (447, 448), are also effective *in vivo*. These compounds are more effective in combination with cefazolin, imipenem, or vancomycin (449). (ii) A synthetic autoinducer of AIP and its derivatives (I to IV) also inhibit RNAIII (447). (iii) Finally, nonfunctional AIP analogues can repress many AgrC receptors (I to IV). These compounds are the most potent described, to date (450). In addition, numerous inhibitors can be highlighted in relation to inhibitory QS in *P. aeruginosa* (221, 451), such as (i) halogenated compounds; (ii) AHL antagonists; (iii) cell extracts and secretion products; (iv) quorum quenchers obtained from plants and food; (v) acylases and lactonases (452); and (vi) an inhibitor of the OmpA protein, which is an important virulence factor (453).

Several authors have studied the use of lytic phage cocktails to prevent biofilm formation by bacteria, such as *S. aureus* and coagulase-negative staphylococci (CoNS), *Enterococcus faecalis*, *E. faecium*, *E. coli*, *Proteus mirabilis*, *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. (454–459). Fernández et al. demonstrated that *S. aureus* biofilms formed at nonlethal concentrations of phage phiIPLA-RODI present a unique physiological state that may benefit both the host and the predator (460). Thus, biofilms may be denser and contain more DNA, depending on phage pressure. Significantly, transcriptome sequencing (RNA-seq) data showed (p)ppGpp response expression, which may slow the movement of the bacteriophage within the biofilm. The result would be an equilibrium that would help bacterial cells to survive environmental pressures while maintaining a reservoir of sensitive bacterial cells available to the phage on reactivation of the latent carrier subpopulation. The study of lysins from phages and bacterial lytic proteins is also of great interest. Several endolysins have been found to exert a lytic function against Gram-positive bacteria, such as *S. aureus* (461) and other bacteria (462). In relation to Gram-negative bacteria, we highlight the endolysin PlyE146, which displays lytic activity against *E. coli*, *P. aeruginosa*, and *A. baumannii* (463). Finally, the LysAB2 endolysin, first described in 2011, shows activity against bacteria, such as methicillin-resistant *S. aureus*, *A. baumannii*, and *E. coli* (464). Interestingly, peptide-induced modification of this endolysin extended the range of lytic activity (465).

Recently, Wood et al. reported the effective use of anticancer drugs, including 5-fluorouracil (5-FU), gallium (Ga) compounds, mitomycin C, and cisplatin, to treat persistent bacterial infections (466–468).

New antibacterial agents against bacterial persisters have been described and include the following. (i) Specific inhibitors of respiration (MenG inhibitors, such as DG70 in *Mycobacterium tuberculosis*) are accepted antitubercular agents. Nevertheless, further studies will be necessary to optimize the route toward the proposal of candidates for validation of *in vivo* efficiency (469). In addition, analysis of physiological, biochemical, and pharmacological data shows that cytochrome *bd* plus the biosynthetic pathways of menaquinone, fumarate dehydrogenase, hydrogenase, and ubiquinone dehydrogenase are potential targets for the next generation of drugs (470). (ii) Suramin is an inhibitor of the RecA protein and the SOS response in *M. tuberculosis* (471). (iii) 3-(4-[4-Methoxyphenyl] piperazin-1-yl) piperidin-4-yl biphenyl-4-carboxylate wakes persisters, although the procedure has not been determined (472, 473). (iv) The antibiotic acyldepsipeptide ADEP4 eliminates ATP demands by ClpP protease activation, producing the death of persister cells (474). (v) Sytox Green NH125 acts against MRSA persisters by permeabilizing the bacterial membrane (475). (vi) Pyrazinamide (an

analogue of nicotinamide) is used to treat *M. tuberculosis* and *Borrelia burgdorferi* infections. This compound acts by disrupting *trans*-translation during retrieval of the halted ribosome process (476). (vii) Daptomycin has also been associated with the killing of *B. burgdorferi* persisters (477). (viii) The compound *cis*-2-decenoic acid (CDA) reduces biofilm-derived *P. aeruginosa* persister cells (478). (ix) Pretreatment of *Burkholderia cenocepacia* biofilms with itaconic acid, an isocitrate lyase inhibitor, produced lower survival of persisters in a *Burkholderia cenocepacia* biofilm in response to a treatment including tobramycin (83). (x) The use of subinhibitory concentrations of 2′,3,4′,5,7-pentahydroxyflavone, tetrahydropyrrole, and quercetin, alone or in combination with antibiotics, can prevent or control biofilm formation. Synergetic interactions with antibiotics have been observed to affect biofilms of *S. aureus* for strain SA1199B overexpressing NorA. Culture of this strain with ciprofloxacin at subinhibitory concentrations led to acquisition of tolerance to the antibiotic. However, this was reversed when 2′,3,4′,5,7-pentahydroxyflavone and quinine were added, demonstrating that the inclusion of phytochemicals in combined therapies improves treatments and decreases antibiotic resistance, strongly affecting *S. aureus* in biofilm and planktonic states (479). (xi) Finally, small molecules which participate in the LexA autoproteolysis step in the SOS system may be used as SOS inhibitors and administered as adjuvants to current antibiotics (204).

CONCLUSIONS

Obtaining information about the metabolism of cells with tolerance and persistence phenotypes is challenging but also provides valuable tools for the development of antitolerance and/or antipersistence compounds. Interestingly, in-depth analysis of these mechanisms has revealed a larger number of data for gastrointestinal pathogens than for respiratory ones, possibly due to differences in environmental and antimicrobial pressures. The knowledge of the molecular mechanisms of tolerant populations and/or persistent subpopulations is key to the fight against multidrug-resistant (MDR) bacteria. The current lack of effective antibiotics against MDR pathogens drives the need to develop new bacterial treatments, which must include tolerant and persister bacterial cells as targets due to the relationships among these bacterial populations.

Moreover, phenotypic detection of tolerant populations and persistent subpopulations in microbiological clinical practice through MDK and TDtest measurements would help to (i) improve the guidelines for anti-infective treatments (selection as well as duration of treatment), (ii) avoid the evolution or maintenance of resistant bacterial populations, (iii) allow for study of the antitolerance and antipersistence ability of antimicrobials used in clinical practice, and (iv) allow for analysis of the efficiency of new anti-infective treatments (including antitolerance and/or antipersistence ability).

In conclusion, phenotypic, molecular, and clinical studies of these bacterial populations are important for the development of new anti-infective treatments efficient in the fight against MDR pathogens. Research on the diagnosis and treatment of infection according to bacterial populations, host environments, and patient features may become essential for the development of personalized medicine.

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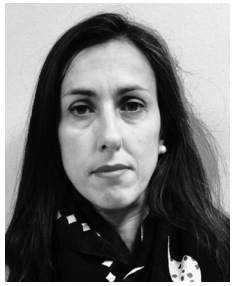
L. Fernández-García obtained her degree in biology from Oviedo University (2007 to 2012) and her master's degree in cellular, molecular, and genetic biology from A Coruña University (2014 to 2015). She is currently undertaking Ph.D. research at the Institute for Biomedical Investigation-A Coruña University Hospital Complex (INIBIC-CHUAC) as a member of the microbiology group, under the supervision of M. Tomás. She is currently the recipient of a Xunta de Galicia Ph.D. student contract. Her Ph.D. research focuses on mechanisms of persistence and tolerance in nosocomial pathogens. She has published nine articles in scientific journals and has also published one book chapter and has another in press.



L. Blasco obtained her Ph.D. in biotechnology from the University of Santiago de Compostela (USC) in 2011. She began her research career while undertaking a master's degree program in biotechnology at the USC. She has participated as a member of the biotechnology group of the Microbiology and Parasitology Department of the USC, working on several projects within the field of industrial microbiology. Since 2015, she has been carrying out postdoctoral research as part of the microbiology group led by M. Tomás at the Institute for Biomedical Research-A Coruña University Hospital Complex (INIBIC-CHUAC), Spain. Her main research interest is the search for new treatments for use in the fight against multiresistant bacteria (bacteriophages, endolysins, and quorum sensing inhibition).

A. Ambroa is currently pursuing a Ph.D. degree at the University of A Coruña (UDC) and has been undertaking research at the Institute for Biomedical Research, A Coruña (INIBIC), Spain, since September 2017. He obtained his degree in biotechnology from the Rovira i Virgili University (Tarragona, Spain) in 2016. He has completed internships at the Pontevedra Provincial Hospital (Pontevedra, Spain; June 2015), the Sant Joan de Reus University Hospital (Reus, Spain; January to March 2016), and the Faculty of Medicine and Health Science of the Rovira i Virgili University (Reus and Tarragona, Spain; March to June 2016). In 2017, he was awarded a master's degree in clinical investigation (with a specialty in clinical microbiology) from the University of Barcelona. During the master's degree course, he also completed another internship, at the Vall d'Hebron Hospital (Barcelona, Spain; January to June 2017). He is currently investigating the role of the type VI secretion system (T6SS) in multidrug-resistant bacteria in relation to virulence and resistance mechanisms, under the supervision of M. Tomás.

M. L. Pérez del Molino obtained a Ph.D. in the area of chemical physics from the University of Santiago de Compostela (USC), Galicia, Spain, with a specialty in clinical microbiology and parasitology at the University Clinical Hospital of Santiago de Compostela (1980 to 1983). Since 2016, she has been Head of the Microbiology and Parasitology Department of the University Clinical Hospital of Santiago de Compostela. She previously worked as an Assistant Professor in the Department of Microbiology and Parasitology of the USC (1984 to 1986). Subsequently, she worked as a Clinical Microbiologist in the area of diagnosis of respiratory pathology, focusing on tuberculosis disease (1988 to 2016), and as Head of the reference laboratory of mycobacteria in Galicia (1998 to 2018). She has collaborated with the WHO on studies of resistance to antituberculosis drugs; her work features over 60 publications about microbiological diagnosis, epidemiology, and antimicrobial resistance of mycobacteria and other respiratory pathogens.

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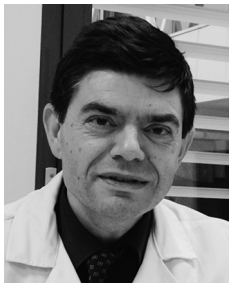

G. Bou received his Ph.D. from the Molecular Biology Center (CSIC)-Autonoma University, Madrid, Spain. He also completed a residence in clinical microbiology at the Ramon y Cajal Hospital. Afterwards, he was granted a postdoctoral position with the Fulbright Scholarship program to work in the Laboratory of Medicine at Mayo Clinic, Rochester, MN. Dr. Bou then served as an Investigator at the National Health System in Spain (2001 to 2005) and as a consultant in clinical microbiology (2005 to 2010), and at present, he is the Head of the Microbiology Department of the University Hospital A Coruña (CHUAC). Since 2009, he has been an Associate Professor of medical microbiology at the University of Santiago de Compostela. Dr. Bou's research focuses on understanding the molecular basis for antimicrobial resistance in human pathogens, developing rapid tests for detecting resistant organisms, and designing and developing bacterial vaccines. So far, he has published more than 200 international peer-reviewed papers on these topics, as well as obtaining 7 related patents. Recently, he was conferred the honorary title of ESCMID Fellow for professional excellence and service to society.

R. García-Contreras has been an Associate Professor in the Microbiology and Parasitology Department of the Medicine Faculty of the National Autonomous University of Mexico (UNAM) since 2014. From 2010 to 2014, he was an Associate Professor at the National Institute of Cardiology. In 2005, he obtained his Ph.D. at UNAM. He completed two postdoctoral positions, the first in the Department of Chemical Engineering at Texas A&M University, in the group of Thomas K. Wood, working on the genetic basis of biofilm formation in *Escherichia coli*, and the second in the Molecular Cell Physiology Department at the VU University of Amsterdam, with Fred Boogerd, working on *E. coli* central metabolism. Currently, his research is centered on the study of the resistance mechanisms of *Pseudomonas aeruginosa* against antivirulence compounds and novel antimicrobials, the influence of quorum sensing in virulence and bacterial physiology, and the repurposing of drugs to treat multidrug-resistant bacteria.

T. K. Wood is the Endowed Biotechnology Chair and a Professor in the Department of Chemical Engineering at Pennsylvania State University. He was formerly the Northeast Utilities Endowed Chair in Environmental Engineering at the University of Connecticut (1998 to 2005) and the O'Connor Endowed Chair at Texas A&M University (2005 to 2012). He obtained his Ph.D. in chemical engineering from North Carolina State University in 1991 by studying heterologous protein production and obtained his B.S. from the University of Kentucky in 1985. His current research pursuits include understanding the genetic basis of biofilm formation in order to prevent disease and to utilize biofilms for beneficial biotransformations, including remediation, green chemistry, and energy production. He also uses systems biology approaches to understand cell resistance, specifically discerning the roles of toxin-antitoxin systems and cryptic prophages in antibiotic resistance and persistence. He also has utilized protein engineering to control biofilm formation as well as for bioremediation and green chemistry.

M. Tomás, M.D., Ph.D. (and Clinical Microbiologist), has investigated different mechanisms of resistance to antimicrobials in nosocomial MDR pathogens in various research centers, within the framework of the Rio Hortega and Miguel Servet program (ISCIII-SERGAS). She is currently working as the Molecular Microbiology Coordinator in the Microbiology Department of CHUAC and as a Principal Investigator (PI) in the Institute for Biomedical Research (INIBIC-CHUAC), initiating new research on the relationships between resistance, tolerance, and persistence mechanisms in microbial pathogens to improve new anti-infective treatments, such as phage and antivirulence therapies against persister cells. She has over 70 publications and 3 patents related to new anti-infective treatments and molecular techniques and has completed more than 10 projects as PI (5 research projects and 5 innovation projects) (http://www.mariatomas.me/). Finally, she is Guest Editor for several international journals (*Frontiers in Cellular and Infection Microbiology* and *Marine Drugs*) and is a member of ASM, ECCMID, SEIMC, and the REIPI network.