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The Multiple Signaling Systems Regulating Virulence in *Pseudomonas aeruginosa*

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INTRODUCTION

Cell-to-cell communication by means of diffusible signaling molecules allows bacteria to trigger coordinated responses to achieve outcomes that would otherwise remain impossible for individual cells. During the past 2 decades, much attention has been given to bacterial communication systems due to their involvement in acute and chronic infections. Analyses of the molecular mechanisms of cell-to-cell communication may help scientists to develop specific antimicrobial agents that will decrease both the defensive and offensive traits of pathogens. The signaling network of *Pseudomonas aeruginosa* is perhaps one of the most complex systems known and, to date, is the best studied among all microorganism systems. It consists of multiple interconnected signaling layers that coordinately regulate virulence and persistence, driving the emergence of *P. aeruginosa* from the enormous number of species that comprise the biodiverse bacterial domain to join an elite group of a few dozen that pose a major threat to humans. This review summarizes the major signaling systems regulating virulence and persistence in *P. aeruginosa*, with special attention to those involving the production and detection of diffusible signaling molecules. Due to the complexity and diversity of these signaling networks, we define the relevance of each system with regard to signal integration, adaption responses, and virulence, emphasizing the importance of less-well-studied signals as potential key elements in the global virulence network of *P. aeruginosa*. (Gene and protein numbers in this article refer to the corresponding numbers from the *P. aeruginosa* PAO1 genome.)

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THE EMERGING NEED FOR NOVEL AGENTS AGAINST *P. AERUGINOSA* INFECTIONS

Since its initial discovery in the late 19th century (63), the Gram-negative bacterium *P. aeruginosa* has gained a notorious place in the list of infamous human pathogens (65, 158, 206). The arrival of the antibiotic era largely palliated the previously fatal outcome of acute infections in healthy patients. Only a relative improvement has been achieved in the eradication of chronic infections, which develop mainly in individuals suffering from cystic fibrosis or severe burns or who are immunocompromised (30, 74, 98). Two intrinsically related factors in the fatal outcome of infection in these patients are the rapid prescription of not always appropriate antibiotic treatments and the development or acquisition of multidrug-resistant strains. While the use of an appropriate antibiotic(s) has been reported as an essential factor in the eradication of *P. aeruginosa* infections (102, 122, 134), conversely, antibiotic abuse significantly contributes to increasing resistance by exerting a continuous selective pressure for the acquisition of such capabilities. Antibiotics alone do not account for the high prevalence of multidrug-resistant variants: *P. aeruginosa* has multiple, chromosomally encoded intrinsic mechanisms of resistance, including low permeability of the cell envelope and numerous multidrug efflux pumps. Another major factor accounting for the successful invasive behavior and persistence of this bacterium is its high adaptability, allowing rapid colonization of different environments. To perform these adaptations, *P. aeruginosa* has evolved a complex and extensive array of regulatory signaling networks that detect and react to endogenous and environmental molecules, triggering massive changes in genetic expression.

AHLs: THE CLASSICAL GRAM-NEGATIVE SIGNALS

N-Acyl homoserine lactones (AHLs) were the first broadly accepted bacterial cell-to-cell signals to be discovered and, to date, remain the most-studied communication molecules in bacteria (2, 3, 59, 60, 72, 143). A large number of Gram-negative bacteria produce and use these signals to control and regulate gene expression in a cell density-dependent manner known as quorum sensing (QS) (68, 205, 208). In general, AHLs consist of fatty acids, varying in length and substitution, linked via a peptide bond to a homoserine lactone moiety. AHLs are commonly synthesized by members of the LuxI family of proteins and are sensed by members of the LuxR family of transcriptional regulators (69, 70). After a certain concentration of AHLs (the threshold) has been produced (correlating to a certain bacterial cell density), a complex with the cognate LuxR transcriptional regulator will be formed, enabling binding to DNA, thereby altering the expression of multiple virulence genes. Two different AHL systems coexist in *P. aeruginosa*: the Las and Rhl systems. The Las system produces and responds to *N*-3-oxo-dodecanoyl homoserine lactone (3-oxo-C_{12}-HSL), which is produced by the LasI synthase (PA1432) and recognized by the transcriptional regulator LasR (PA1430) (154, 156). The Las system controls the production of multiple virulence factors involved in acute infection and host cell damage, including the LasA (PA1871) and LasB (PA3724) elastases, exotoxin A (PA1148), and alkaline protease (PA1246) (73, 99, 154, 191). The second AHL system, the Rhl system, produces and responds to *N*-butanoyl homoserine lactone (C_4-HSL) (157). This molecule is generated by the RhlI synthase (PA3476) and sensed by the transcriptional regulator RhlR (PA3477), inducing the expression of several genes, including those responsible for the production of rhamnolipids, and repressing those responsible for assembly and function of the type III secretion system (T3SS), a major virulence determinant in human infections that allows the release of toxic proteins into the cytoplasm of eukaryotic cells (12). A hierarchical relationship exists between the Las and Rhl systems: the Las system controls the Rhl system, as the 3-oxo-C_{12}-HSL–LasR complex directly upregulates *rhlR* transcription (112). Thus, activation of the LasIR system allows the later activation of the RhlIR system (Fig. 1). Experiments with mouse models demonstrated that deletion of either AHL synthases or AHL receptors results in a decrease in infection severity (155, 172, 183).

Further Regulation of the AHL Systems

In addition to RhlR and LasR, *P. aeruginosa* possesses several putative LuxR-type homologues lacking a LuxI-type cognate partner; these homologues have been designated orphan LuxR homologues (67). Their function and potential relationship to AHL signaling remain unknown, with the exception of QscR (PA1898), which exhibits full conservation with functional LuxR-type proteins and forms complexes with LasR and RhlR. These delay the expression of quorum sensing-regulated genes, thereby reducing bacterial virulence both *in vitro* and *in vivo* (35, 115). Recently, Chugani and Greenberg revealed an even higher level of complexity in *P. aeruginosa* AHL signaling, reporting a set of 37 genes whose expression was controlled by AHLs in the absence of LasR, RhlR, and QscR (34). These recent results raise questions about AHL quorum sensing regulation. What is the identity and mechanism of the protein(s) or recognition factor for these AHL signals? Are these mechanisms present in other species? Does this system function by recognizing native AHL signals only, or does it respond to AHLs produced by other bacteria and is thus involved in interspecies communication?

Given this complexity, it seems obvious that AHL quorum sensing must be tightly regulated to coordinate the correct time and place of expression of virulence factors. Three regulators have been found in *P. aeruginosa* that contribute to the timing and level of control of AHL-regulated virulence. The first, RsaL (PA1431), acts as a major transcriptional repressor of the Las system, controlling the maximal levels of AHLs, and also therefore virulence factors, produced (42). RsaL binds simultaneously with the 3-oxo-C_{12}-HSL–LasR complex to the *lasI* promoter (163), inhibiting its transcription, while in parallel it controls the repression of AHL-related virulence by directly binding to the promoters of pyocyanin and hydrogen cyanide (HCN) genes (165).

Controlling the Activation Threshold

The second regulator, QteE (PA2593), was recently shown to prevent the posttranslational accumulation of LasR by reducing its stability and also blocking RhlR accumulation by an as yet unknown LasR-independent mechanism (179). This activity inhibits the induction of virulence phenotypes, as shown by attenuated infection by *P. aeruginosa* in plant and *Drosophila melanogaster* models upon overexpression of *qteE* (118). QslA (PA1244) prevents early activation of QS-regulated virulence by forming complexes with LasR that prevent its binding to the target DNA. In contrast to QteE, QslA does not affect RhlR QS activation or LasR stability, nor does its absence correlate with an early activation of QS (176). These newly discovered regulators provide a logical explanation for how a threshold for activation can be created in bacteria where early activation of QS-dependent virulence is

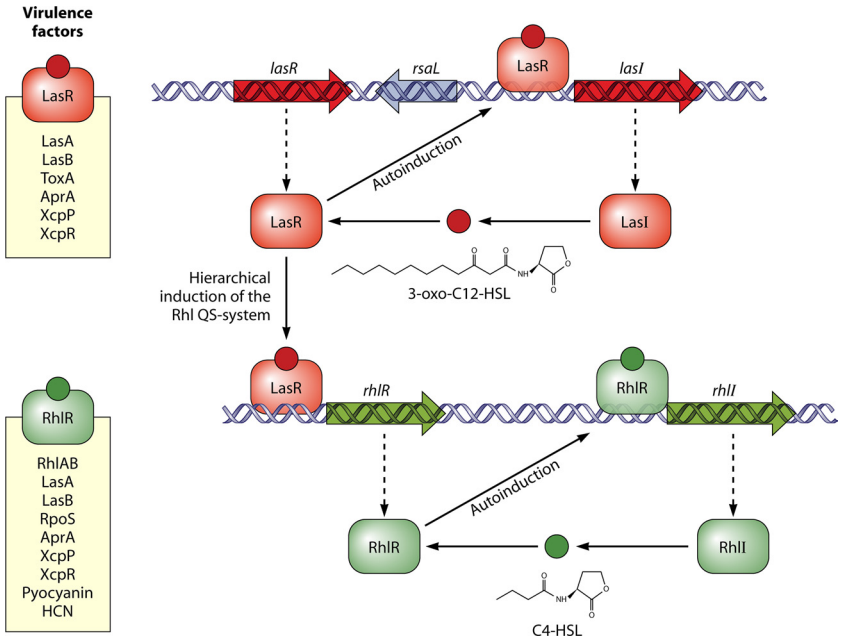


FIG 1 Virulence regulation of and interactions between the two AHL quorum-sensing systems in *P. aeruginosa.* After a threshold concentration of 3-oxo-C_{12}-HSL is produced, the 3-oxo-C_{12}-HSL–LasR complex binds the promoter regions of multiple genes, activating or repressing their transcription. Among the genes upregulated by this complex are *lasI*, which enhances the production of 3-oxo-C_{12}-HSL (autoinduction effect), and *rhlR*, which increases the production of the *rhl* response regulator RhlR, activating the second AHL pathway at an earlier stage. Virulence factors regulated by each respective receptor-ligand complex are detailed on the left.

avoided during the initial growth phase. It remains possible, although not yet proven, that QteE and/or QslA also actively represses AHL-dependent gene expression during late stationary phase to conserve energy when AHL-related virulence is no longer needed.

Additional AHLs in *P. aeruginosa*

The AHL systems discussed here encompass only the two main AHLs of *P. aeruginosa* (C_4-HSL and 3-oxo-C_{12}-HSL), although low concentrations of other distinct AHLs, namely, 3-oxo-C_{14}-HSL and 3-oxo-C_{10}-HSL, can be detected in the supernatants of *P. aeruginosa* cultures (28). The presence of smaller quantities of these AHLs may be due to a case of mistaken identity where the LasI synthase couples the wrong acyl carrier protein (ACP) to *S*-adenosylmethionine (SAM). However, these AHLs might also originate from the action of a different type of AHL synthase. In addition to the LuxI-type synthase, two other unrelated AHL synthase families have been reported: the LuxM synthase family, exclusive to *Vibrio* spp. (7, 77), and the more diverse HdtS synthase (113), originally identified in *Pseudomonas fluorescens*, with putative homologues in several *Pseudomonas* spp., including *P. aeruginosa*.

DKPs AND LuxR ACTIVATION

DKPs

A close examination of LuxI and LuxR homologues in all sequenced genomes of Gram-negative bacteria clearly reveals an increased prevalence of LuxR- over LuxI-type proteins. Whether this ratio reflects a predominance of eavesdroppers over speakers or is an indication of the presence of unidentified alternative LuxR-binding molecules remains a matter of debate. The idea of a novel set of molecules capable of binding and activating LuxR-type proteins was confirmed as a reality in 1999, when Holden and coworkers purified and elucidated several structures of a novel family of cyclic dipeptides, termed diketopiperazines (DKPs), from the supernatants of various bacteria, including *P. aeruginosa* (Fig. 2) (92). DKPs have been proven to interfere with the quorum-sensing systems of various bacteria; this interference is most likely by binding to the LuxR family of receptors, either activating or antagonizing AHL signals. In the same work, the authors identified three different DKPs in the supernatants of various bacteria: cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) in *P. aeruginosa*, *Proteus mirabilis*, and *Citrobacter freundii*; cyclo(ΔAla-L-Val) in *Enterobacter agglomerans*; and cyclo(L-Phe-L-Pro) in *P. fluorescens* and *Pseudomonas alcaligenes*.

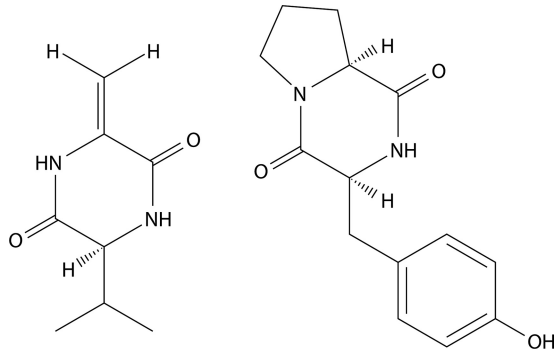


FIG 2 Structures of the two DKPs produced by *P. aeruginosa.*

Despite the discovery of this novel type of signaling compound over 10 years ago, little attention has been given to DKPs. Two possible reasons for this apparent lack of interest are that the high concentrations of DKPs required to activate LuxR-like quorum-sensing systems strengthen the idea of a fortuitous cross talk (91) and the fact that no bacterium has yet been demonstrated to regulate QS solely by depending on DKPs, undermining the importance of these molecules as essential signaling compounds. However, DKPs may become the focus of more intensive research in the coming years due to the increasing number of studies on the additional antimicrobial properties of these compounds.

Additional Properties of DKPs

Lactobacillus plantarum produces two DKPs, namely, cyclo(L-Phe-L-Pro), as found in *P. fluorescens* and *P. alcaligenes*, and cyclo(L-Phe-*trans*-4-OH-L-Pro), and both of these have been reported to display antifungal activity (189). Cyclo(L-Phe-L-Pro) has also been detected in the supernatants of the human pathogens *Vibrio vulnificus*, *Vibrio cholerae*, *Vibrio parahemolyticus*, and other related *Vibrio* spp. (151). An interesting aspect highlighted by the authors is that while this molecule activates the Lux reporter system of *Vibrio fischeri*, in *V. vulnificus* and other related species this DKP enhances the expression of the outer membrane protein *ompU* gene and the cholera toxin *ctxAB* genes, known to be under the control of ToxR. The presence of DKPs in *V. vulnificus* is especially intriguing because this bacterium does not produce AHLs and therefore could use DKPs to activate orphan LuxR regulators. On the other hand, in a recent study on the QS properties and mode of action of DKPs, Campbell and coworkers found no evidence of LuxR-like activation or interaction by any of the DKPs tested (24), including those previously reported by Holden and colleagues. These controversial results cast serious doubts on the role of DKPs as bacterial signaling molecules, and their potential involvement in *P. aeruginosa* QS-regulated virulence requires further research.

4-QUINOLONE SIGNALING: THE *PSEUDOMONAS-BURKHOLDERIA* LANGUAGE

PQS

Despite 4-quinolones having been discovered in the 1940s (82) and subsequently studied due to their antibacterial effects (37, 119), their signaling properties were not reported until more than 50 years later, when Pesci and coworkers identified the first signaling role for a 4-quinolone in *P. aeruginosa* (159). This molecule, 2-heptyl-3-hydroxy-4-quinolone, termed the *Pseudomonas* quinolone signal (PQS), is synthesized from anthranilate and an α-keto-fatty acid by the products of the *pqs* biosynthesis genes *pqsABCD* (PA0996 to PA0999) (18, 61). These synthesize the precursor molecule 2-heptyl-4(1H)-quinolone (HHQ), which is finally converted into PQS by PqsH (PA2587). After a certain threshold concentration of PQS in the extracellular medium is reached, this molecule binds to its cognate receptor, PqsR (also called MvfR [PA1003]). The resulting complex activates the expression of the *pqsABCDE* and *phnAB* (PA1001-PA1002) operons, increasing PQS and pyocyanin production (25, 46, 52). The increase in production of PQS resulting from the PQS-PqsR complex binding to the *pqsA* promoter region constitutes an autoinduction mechanism similar to that observed in AHL quorum-sensing systems. Two additional regulators, MvaT (PA4315) and its homologue MvaU (PA2667), are found in several *Pseudomonas* spp. and may also be involved in PQS production in *P. aeruginosa* (53, 197). An *mvaT* mutant exhibits more production of PA-IL lectin and pyocyanin, reduced biofilm formation and swarming motility, and increased drug resistance (53, 204). The observation that *mvaT* and *mvaU* single mutants increase pyocyanin synthesis while an *mvaT mvaU* double mutant abolishes pyocyanin and PQS production suggests that these regulators work in different ways to control pyocyanin production, with one involving PQS production and the other directly controlling pyocyanin synthesis (117). In addition to the four genes involved in PQS biosynthesis, the *pqsABCDE* operon contains a fifth, *pqsE* (PA1000), encoding a protein with a metallo-β-lactamase fold (62) that is not required for PQS synthesis (71). Despite the recent elucidation of the crystal structure of PqsE (215), little is known about its function and natural substrate. PqsE is the major virulence effector in the 4-alkyl-quinolone (4-AQ) system, controlling the production of several virulence factors, such as pyocyanin, lectin, rhamnolipids, and HCN (52, 71), which are all implicated in toxicity and acute infection (Fig. 3). The 4-quinolone signaling system is linked in a hierarchical manner to the AHL signaling systems of *P. aeruginosa*, as LasR (positively) and RhlR (negatively) control the levels of PQS by binding to the promoter region of the PqsR regulator (202). Additionally, PqsE alone is sufficient to regulate its virulence target genes via the *rhl* QS system intrinsically linked to RhlR (62). Mutations in either *pqsA* or *pqsE* significantly reduce *P. aeruginosa* virulence in plant and animal infection models (46, 164).

Function of HHQ as a Signal

Although PQS is the major 4-quinolone signaling molecule produced by *P. aeruginosa*, approximately 50 structurally related 4-quinolones are also produced by the PqsABCD proteins. Most of these molecules are produced in amounts too small to play a significant role in cell-to-cell signaling. However, one of them, the PQS precursor HHQ, has clearly been demonstrated to also act as a cell-to-cell signal (47). HHQ can be released into the extracellular medium and subsequently taken up by neighboring cells, in which it either is converted into PQS by PqsH or binds directly to PqsR, in both cases activating PQS-regulated gene expression to levels similar to those observed in response to PQS itself (with the only exception being the *phzA1-phzG1* [PA4210 to PA4216] operon, responsible for pyocyanin biosynthesis, which is activated by PQS but not by HHQ) (211). Although a study of *P. aeruginosa* PAO1 revealed that HHQ conversion to PQS is necessary for driving the expression of the lectin gene *lecA* (PA2570) (51), studies using a *pqsH* mutant in *P. aeruginosa* UCBPP-PA14 indicated that the conversion of HHQ into PQS is unnecessary, as HHQ alone was able to fully activate PqsR-dependent virulence expression (211). A role of HHQ in cell-to-cell signaling is even more evident if we take into account that only *P. aeruginosa* produces PQS, while other *Pseudomonas* spp. and *Burkholderia* spp. rely on HHQ and other methylated 4-hydroxy-2-alkylquinoline analogues for 4-quinolone signaling (50, 199).

The Additional Role of PQS

Since PQS seems to be dispensable for cell-to-cell signaling, what is the benefit to *P. aeruginosa* of having an enzyme (PqsH) to generate this molecule? Recent studies on PQS may have solved this question, demonstrating that the presence of the additional

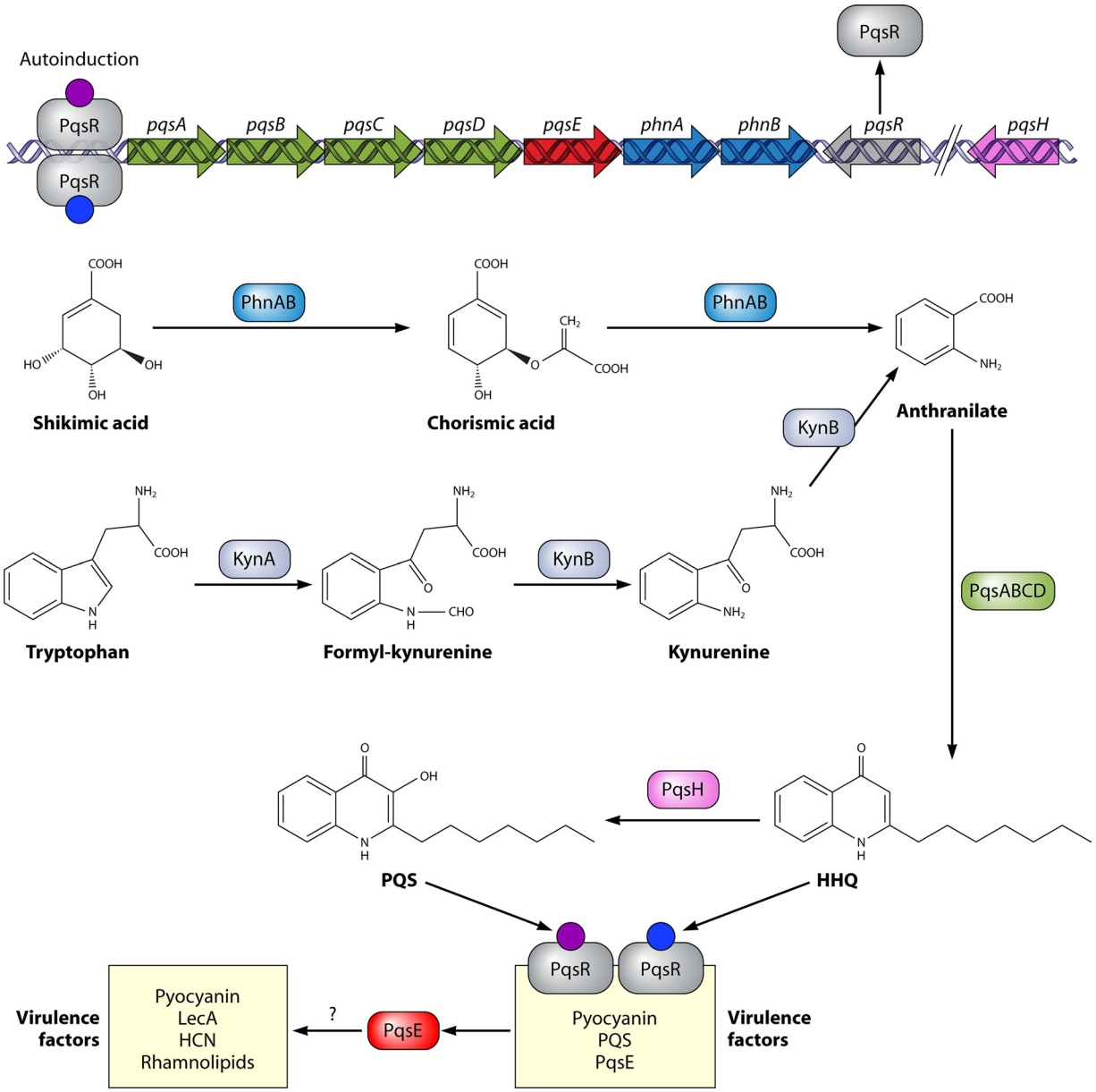


FIG 3 Biosynthesis, autoinduction, and virulence regulation by 4-alkyl-quinolones in *P. aeruginosa*. Biosynthesis of PQS starts with the conversion (by the PqsABCD proteins) of anthranilate (which originates from either the kynurenine pathway or the PhnAB anthranilate synthase) into HHQ, which is finally converted into PQS by the PqsH monooxygenase. Both HHQ and PQS bind the PqsR regulator, and the complex activates the transcription of the *pqsABCDE* and *phnAB* operons, increasing the levels of PQS (autoinduction) and pyocyanin production. Additionally, transcription of the PQS operon results in an increase in the levels of PqsE, an enzyme of uncharacterized function that increases the levels of pyocyanin, lectin, HCN, and rhamnolipids.

hydroxyl group in PQS is essential for the binding of iron (17). The formation of PQS-iron complexes confers iron-chelating properties upon this molecule; however, iron-bound PQS is not transported back into the cell and therefore does not function as a siderophore (51). Instead, PQS is found at high concentrations in the outer cell membrane of *P. aeruginosa*, within membrane vesicles (128, 129). The presence of iron-bound PQS at the membrane may contribute to the accumulation of iron in close proximity to the cell, facilitating the work of the actual siderophores pyoverdine (PVD) and pyochelin. This mechanism would allow the bacterium to rapidly and efficiently obtain iron without losing siderophores in the surrounding environment (51). Additionally, PvdS, the major regulator of pyoverdine biosynthesis, has been proven to play a role in PQS synthesis by controlling the expression of PqsR (147), demonstrating an intrinsic relationship of PQS production with iron levels.

THE GAC SYSTEM: CONNECTING VIRULENCE, BIOFILM FORMATION, AND SWARMING

The Transition from Acute to Chronic Infection

In addition to the AHL and PQS systems, *P. aeruginosa* controls its lifestyle (free-living and biofilm) and the production of multiple virulence factors via two-component signal transduction systems.

These systems act through phosphorylation cascades that induce conformational changes in regulatory proteins, resulting in global changes in gene expression. Over 60 two-component systems have been found in the genome of *P. aeruginosa*; among them, the GAC system (global activator of antibiotic and cyanide synthesis) is perhaps the most interesting and best studied. Initially identified in *Pseudomonas syringae* in 1992 (93), the GAC system in the genus *Pseudomonas* has gained special notoriety due to the rich metabolic pool produced by the species of this genus and its involvement in microbe-host interactions. Particularly in *P. aeruginosa*, one of the major features attracting researchers is the main role of the GAC system in the transition from acute to chronic infection. In patients suffering from cystic fibrosis, it is not the acute infection mode of *P. aeruginosa* that poses the major threat but the highly resistant biofilm lifestyle that leads to recurrent infections ending in fatal lung failure. The GAC system consists of a transmembrane sensor kinase, GacS (LemA [PA0928]), which upon autophosphorylation transfers a phosphate group to its cognate regulator, GacA (PA2586), which in turn upregulates the expression of the small regulatory RNAs RsmZ (PA3621.1) and RsmY (PA0527.1). Binding of RsmZ and RsmY to the small RNA-binding protein RsmA (PA0905) activates the production of genes involved in biofilm formation and represses multiple genes involved in acute virulence and motility. As a consequence, in a mouse model of acute pneumonia, a mutation in RsmA reduced colonization during the initial infection stages but ultimately favored chronic infection (140).

Multiple Kinases Interact with the GAC System

Two further sensor kinases, LadS (PA3974) and RetS (PA4856), have been found to modulate gene expression via GacA. LadS (lost adherence sensor) acts in parallel to GacS, positively controlling the expression of the *pel* operon (PA3058 to PA3064), which increases biofilm production, and repressing the expression of genes involved in the T3SS (198). The third sensor kinase involved in this pathway, RetS (regulator of exopolysaccharide and type III secretion), controls GacA in an opposite manner to GacS and LadS, promoting acute infection and repressing the expression of genes associated with biofilm production. This was evidenced by a comparison of gene expression in *retS* and *ladS* mutant strains, which clearly demonstrated reciprocal control of the same set of genes (198). Furthermore, tests of the effects of these additional kinases in a mouse model of acute pneumonia revealed that unlike its parental strain, a *P. aeruginosa retS* mutant was unable to establish infection (78).

Interestingly, it was found that the effects of RetS on GacA-dependent virulence expression are not due to a phosphorylation cascade, as expected from the protein function, but to a direct interaction between RetS and GacS. In an elegant study, Goodman and coworkers demonstrated that the formation of heterodimers between RetS and GacS blocks the autophosphorylation ability of the latter, interfering with the consequent phosphotransfer to GacA and leading to a reduction in RsmZ expression. While RsmA bound to RsmZ or -Y promotes the expression of genes involved in biofilm formation, at low concentrations of RsmZ, RsmA promotes the expression of genes involved in acute virulence and represses the expression of genes involved in chronic infections (Fig. 4) (79). The GAC system also has a control on the AHL system via RsmA, by negatively controlling the synthesis of C_4-HSL and 3-oxo-C_{12}-HSL and of extracellular virulence factors controlled by AHLs (105, 160, 168). Furthermore, a recent study published by Filloux and coworkers (137) revealed that the RetS-dependent switch between T3SS and T6SS activities, associated with the transition to chronic infections, is regulated via cyclic di-GMP (c-di-GMP) signaling. This finding reveals how these two pathways regulate a common set of phenotypes, predicting an exciting series of studies that will ultimately aim to unravel the exact molecular mechanism of this interaction.

Despite extensive study of the GAC system, the identities of the signals triggering the phosphorylation response remain unknown. Finding the activators of these sensor kinases has been a long quest for many scientists aiming to ultimately control the behavior of such a relevant bacterial genus. Identifying these signals could provide physicians and biotechnologists with a molecule capable of switching bacterial lifestyles to ease antibiotic treatment during human infections or to control antibiotic production in beneficial species enhancing crop protection.

PIGMENTED SIGNALS: THE COLORFUL LANGUAGE OF *PSEUDOMONAS*

Pyoverdine

Signaling under low-iron conditions. PVDs are the major iron-chelating molecules (siderophores) of *P. aeruginosa* (Fig. 5). The production of pyoverdine involves the production of multiple proteins and is therefore likely to be of considerable metabolic burden to the bacterium, perhaps providing a positive selection pressure for further exploitation of this molecule in other systems. Pyoverdine has been found to initiate a signaling cascade responsible for the production of several virulence factors, including exotoxin A (ToxA), PrpL endoprotease (PA4175), and pyoverdine itself (111).

This signaling cascade involves iron-bound pyoverdine (Fe-PVD), the cell surface receptor protein FpvA (PA2398), and the anti-sigma factor FpvR (PA2388). Upon binding to iron, the Fe-PVD–FpvA complex interacts with the periplasmic domain of FpvR, allowing the expression of the regulators PvdS (PA2426) and FpvI (PA2387). PvdS upregulates the production of ToxA, PrpL, and pyoverdine, whereas expression of FpvI generates a positive-feedback loop through increased production of the PVD receptor FpvA (9). Recently, Shirley and Lamont characterized an additional protein necessary for the transport and signaling cascade of Fe-PVD (178). TonB1 (PA5531) is a member of a family of proteins responsible for the energy transduction required for the import of molecules via outer membrane proteins. Binding of TonB1 to the TonB box present in FpvA is essential for both the transport of Fe-PVD and the consequent signaling cascade controlled by this molecule (Fig. 6). While the role of the PVD signaling pathway *per se* has not been tested *in vivo* in a *P. aeruginosa* infection model, iron availability and iron chelation via PVD play important roles in infection establishment (5, 180) and the development of chronic infections (141).

Phenazines

Phenazines are pigmented, redox-active, heterocyclic, nitrogen-containing molecules secreted by a considerable number of bacteria, including multiple fluorescent *Pseudomonas* spp. Phenazines display a broad spectrum of (toxic) activity toward prokaryotic and eukaryotic organisms, varying according to the nature and position of the substituents on the heterocyclic ring

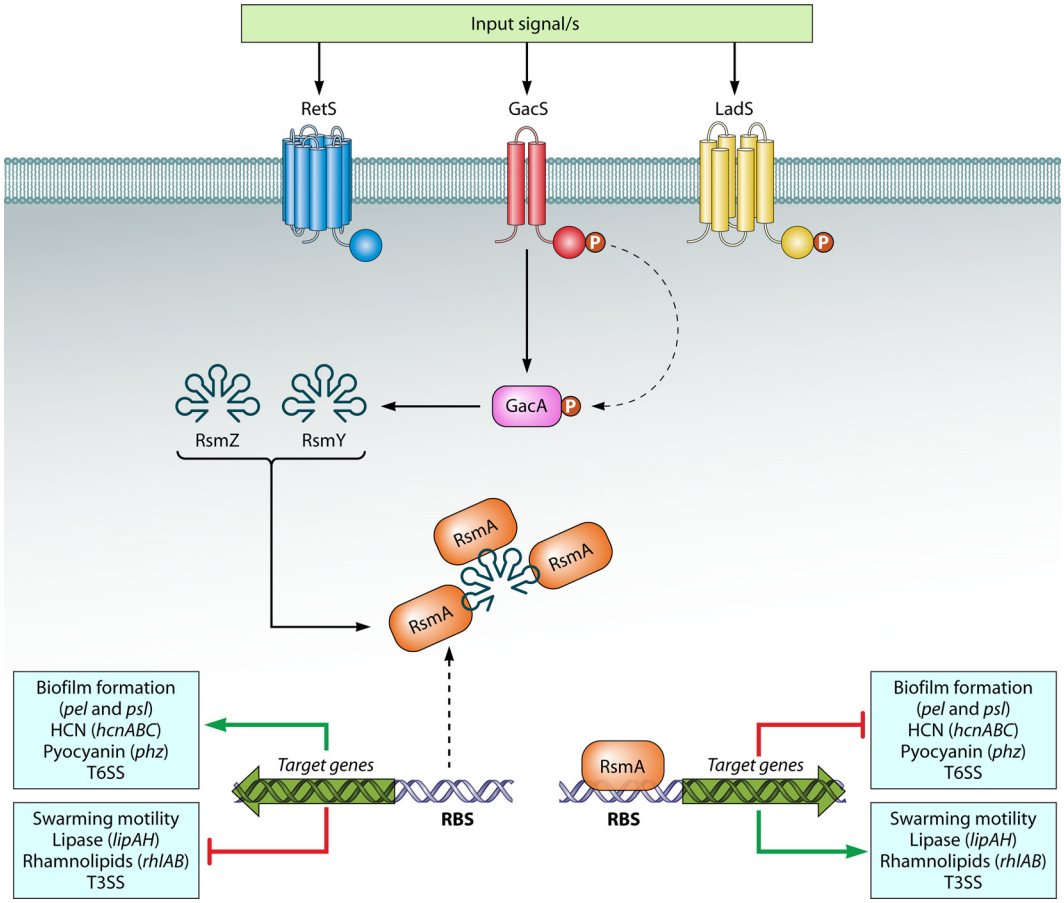
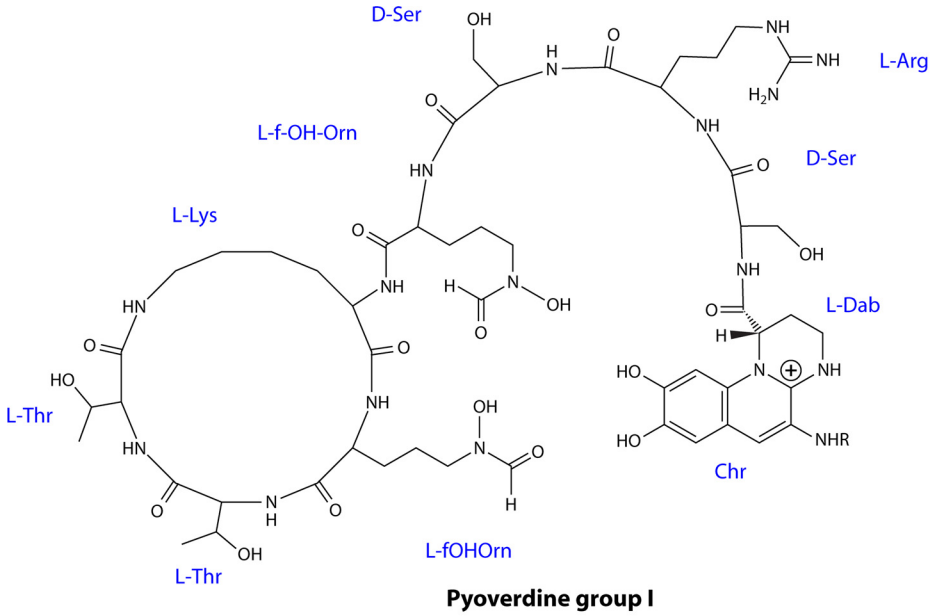


FIG 4 The GAC system network in *P. aeruginosa* controls the reversible transition from acute to chronic infections. The small regulatory protein RsmA binds to the promoters of multiple genes, enhancing bacterial motility and activating the production of several acute virulence factors while repressing the production of virulence factors associated with chronic infections. GacA phosphorylation via GacS stimulates the production of the small RNAs RsmZ and RsmY, which bind to the RsmA protein, releasing the repression of virulence factors associated with chronic infections and repressing the production of acute infection-associated factors. The sensor kinase LadS works in parallel to GacS, activating RsmZ and RsmY production, while the sensor kinase RetS acts in an opposite manner to LadS and GacS, forming a protein-protein complex with GacS that blocks RsmY and RsmZ production.

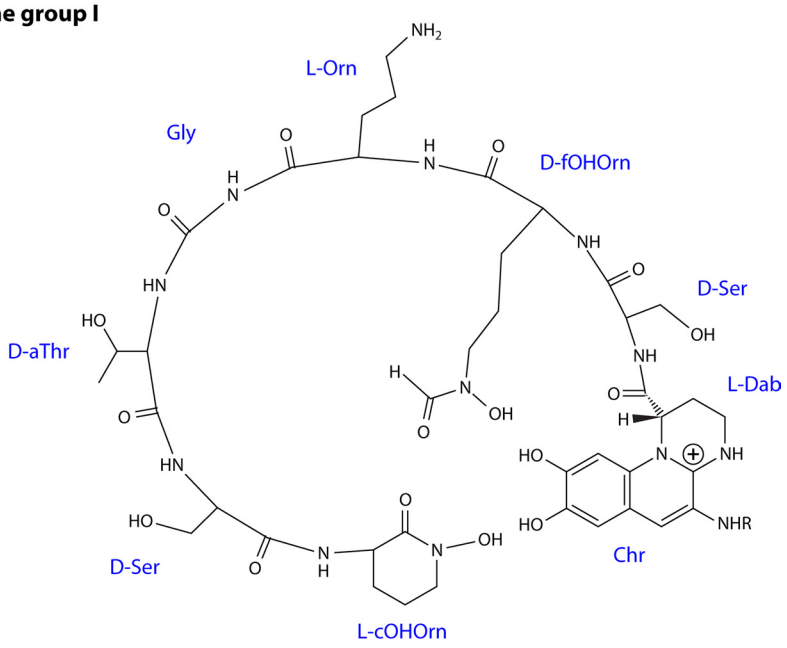
(21, 114, 130). This toxicity confers a clear advantage to phenazine producers by eliminating competitors and enhancing survival in highly populated environments such as the rhizosphere (131). Pyocyanin (5-*N*-methyl-1-hydroxyphenazine), the first and most-studied member of the phenazine family, is produced only by *P. aeruginosa* (Fig. 7), a specificity that has been useful in the rapid diagnosis of this opportunistic pathogen (63, 76, 100). This blue phenazine is one of the major virulence factors in this pathogen, contributing to both acute and chronic infections (123, 207), as it suppresses lymphocyte proliferation (146), damages epithelial cells as a consequence of hydroxyl radical formation (20, 207), inactivates protease inhibitors (consequently causing tissue damage by endogenous proteases) (19), and targets multiple cellular functions (44, 101, 139, 166, 185, 196).

Pyocyanin as a terminal signal. Besides its major function as a virulence factor and electron transfer facilitator (86), pyocyanin also serves as a signaling molecule in *P. aeruginosa*, controlling a limited set of genes, termed the PYO stimulon (48), during the stationary growth phase. This includes genes involved in efflux and redox processes, as well as iron acquisition genes. The efflux pump genes *mexGHI-opmD* (PA4205 to PA4208) and the putative monooxygenase gene PA2274 are among the genes most strongly regulated by the PYO stimulon. These genes were initially identified as part of the PQS regulatory cascade and were assumed to be controlled directly by PQS and PqsE (PA1000) (46). This assumption, however, can now be attributed to the facts that PQS and PqsE directly control phenazine biosynthesis and that pyocyanin itself is responsible for the upregulation of these genes, pointing once again to the complexity of the signaling networks present in this bacterium. The mechanism behind pyocyanin-controlled upregulation of many genes, including *mexGHI-opmD*, PA2274, and PA3718 (encoding a putative MFS transporter), has been demonstrated to occur via the transcriptional regulator SoxR (PA2273). The absence of *sox* boxes in the remaining genes of the PYO stimulon indicates that additional regulatory factors in this signaling cascade remain to be elucidated (48). Similar to the case for PVD, the main role of PYO in an alternative pathway has limited studies on the secondary role of these molecules in signaling.

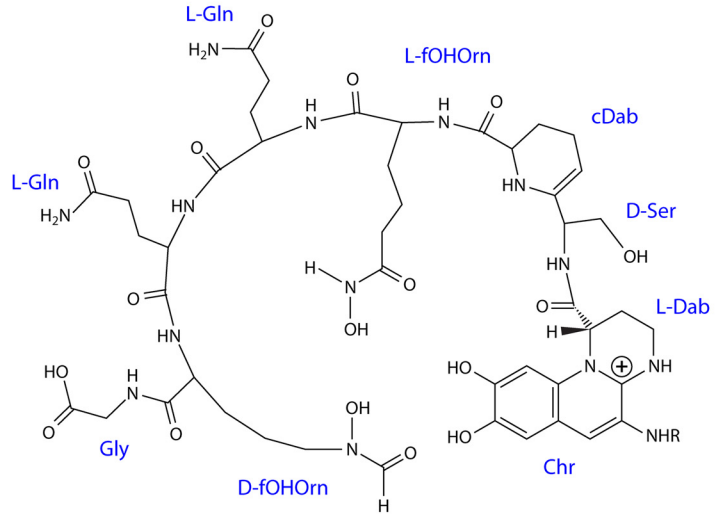
Sox box and colony morphology. Two years after the initial discovery of the signaling role of pyocyanin, Dietrich and coworkers linked pyocyanin levels to the development of wrinkled colonies, a clear morphotype taking place in the late growth phase (49). While wild-type *P. aeruginosa* colonies developed a severely wrinkled phenotype 4 days after inoculation, a phenazine-null



Pyoverdine group I



Pyoverdine group II



Pyoverdine group III

FIG 5 Pyoverdines produced by *P. aeruginosa*. Each *P. aeruginosa* strain produces one type of pyoverdine exclusively. The amino acids D-Tyr and L-Glu (bottom right of each PVD) are further modified during biosynthesis to yield the final pyoverdine. D-Tyr is converted into catechol, and L-Glu into either succinyl, succinamide, ketoglutaryl, or a free acid, and is thus represented by "-R".

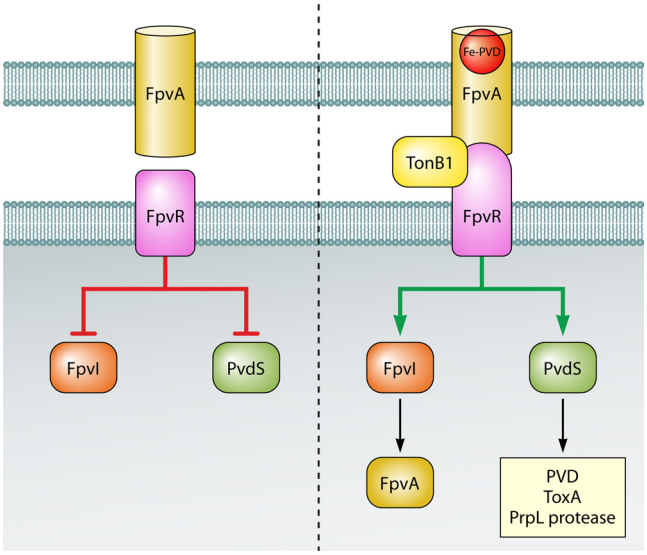


FIG 6 PVD signaling pathway in *P. aeruginosa*. In the absence of Fe-PVD (left), the signaling system is inactive. Binding of Fe-PVD to the PVD receptor FpvA (right) initiates a signaling cascade that requires TonB1 and FpvR and stimulates the production of FpvA, PVD, ToxA, and the PrpL protease.

mutant developed the same type of colonies after only 2 days. In contrast, a wild-type strain overproducing phenazines remained smooth throughout the 6 days of the experiment. The same results were obtained with a *soxR* mutant and a *mexGHI-opmD* mutant, indicating a possible role of high intracellular phenazine levels in colony morphology regulation. The authors also demonstrated that the Gram-positive bacterium *Streptomyces coelicolor*, which contains a *soxR* homologue as well as genes controlled by *sox* boxes, develops a similar colony morphotype and controls gene expression in response to the presence or absence of the two pigmented antibiotics actinorhodin and undecylprodigiosin. These data clearly imply that a conserved Sox-dependent transcriptional regulatory role exists for redox-active pigments in later developmental stages.

DIFFUSIBLE SIGNAL FACTORS (DSF-LIKE FACTORS): FATTY ACIDS AS INTERKINGDOM MESSENGERS

Cross-Kingdom Signaling

Considering the enormous biological diversity present in an ecological niche, it would be naïve to assume that bacterial communication is limited to intraspecies or interspecies signaling. Given the large number of possible interactions in a microcosmos and the high level of competition among organisms, it seems logical that bacteria would produce or receive signals enabling communication with fungi, plants, and animals. In recent years, various signals capable of accomplishing this function have been found. One interesting example of an interkingdom signal interaction was discovered between *P. aeruginosa* and the opportunistic fungal pathogen *Candida albicans*. These two organisms share ecological niches, and both produce signals capable of interfering with the production of virulence factors by the other. Production of 3-oxo-C_{12}-HSL by *P. aeruginosa* inhibits *C. albicans* filamentation, a crucial virulence adaptation for the development of opportunistic infections (90), while production of the fungal metabolite farnesol reduces PQS and pyocyanin levels and swarming motility in *P. aeruginosa* (39, 132). The slight structural resemblance (C_{12}

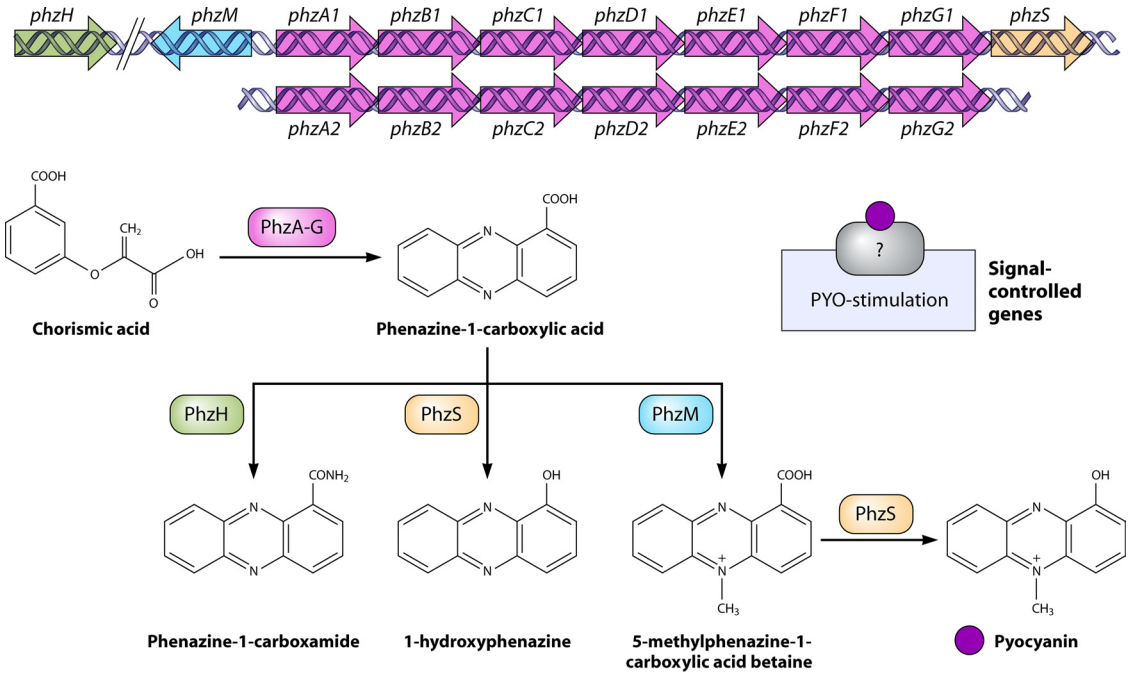


FIG 7 Biosynthesis and signaling system of pyocyanin. Chorismic acid is transformed via the PhzA to -G proteins into phenazine-1-carboxylic acid, which is subsequently converted into different phenazines by the enzymes PhzH, PhzS, and PhzM. The product of the latter is transformed by PhzS into pyocyanin (PYO). Next to its role as a virulence factor, PYO acts as a signaling molecule activating a limited set of genes termed the PYO stimulon. A large fraction of the PYO stimulon genes are controlled by the regulator SoxR, although the mechanism by which PYO activates SoxR, as well as the activation mechanism of SoxR-independent genes, remains unknown.

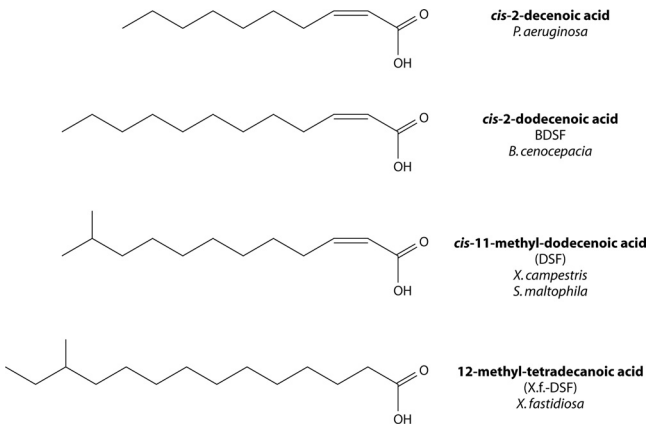


FIG 8 DSF-like fatty acids controlling cell-to-cell signaling in various Gram-negative bacteria.

backbone) between the two molecules suggests that the deleterious effects could be due to a competitive inhibition of the organism's native receptor by the foreign signal molecule. The importance of the fatty acid backbone structure of 3-oxo-C_{12}-HSL and farnesol gained even more relevance with the discovery of certain long-chain fatty acids as intra-/inter- and cross-kingdom signaling molecules.

DSF Signals

The first member of the DSF signal family, *cis*-11-methyl-2-dodecenoic acid, was initially discovered in the plant pathogen *Xanthomonas campestris* and termed diffusible signal factor (DSF) because of its ability to cross cell membranes by passive diffusion (6). In *X. campestris*, DSF controls the production of multiple virulence factors and cyclic glucan, and additionally, it induces biofilm dispersal (6, 57, 200); signaling occurs via a set of genes called *rpf* (regulation of pathogenicity factors) genes. DSF synthesis is carried out by the long-chain fatty acyl coenzyme A (CoA) ligase RpfB and the enoyl-CoA hydratase-like enzyme RpfF (6). The DSF network involves multiple proteins, including RpfG, a two-component regulator containing a novel c-di-GMP hydrolytic domain (HD-GYP) (174), indicating that the DSF signaling circuit is a complex regulatory system with overlapping regulatory layers. A complete overview of DSF and c-di-GMP interactions in *X. campestris* signaling is provided by excellent reviews by Dow et al. and He and Zhang (58, 83).

Although the DSF signaling cascade has been studied extensively in *X. campestris*, these signals are not limited to members of the genus *Xanthomonas*. Various DSF-related molecules have been found in other bacteria: DSF signaling controls motility, lipopolysaccharide (LPS) production, and cell aggregation in *Stenotrophomonas maltophila* (64) and virulence and insect transmission in *Xylella fastidiosa* (29), and the DSF-like signal *cis*-2-dodecenoic acid (BDSF) regulates multiple virulence factors and positively controls biofilm formation in members of the *Burkholderia cepacia* complex (Bcc) (14, 43). Similar to 3-oxo-C_{12}-HSL, DSF-related signals are able to disrupt *C. albicans* filamentation, thus emerging as a novel class of potential antifungal agents (14).

DSF and biofilms. The DSF-like molecule *cis*-2-decenoic acid was isolated from cell-free supernatants of *P. aeruginosa* (Fig. 8). Similar to BDSF in *B. cepacia*, this signal affects biofilm formation and, more interestingly, induces the dispersion of established *P. aeruginosa* biofilms as well as biofilms from a wide range of species (41). As yet, little is known about the synthesis and signaling network of this DSF-like molecule. Given the similarity of the phenotypes observed in bacteria producing DSF signals and the cross-reactivity of these signals, it is tempting to speculate that these compounds may interact with a common substrate-binding domain present in RpfC homologues. In accordance with this hypothesis, mixed-species biofilm experiments using *S. maltophila* and *P. aeruginosa* revealed substantial differences in the architecture of *P. aeruginosa* biofilms and an increase in resistance of this bacterium toward polymyxin antibiotics in the presence of DSF (173). Additionally, it was demonstrated that the sensor kinase PA1396 is essential for the response to these signals. Given the importance of this opportunistic pathogen in human infections and the relevance of antibiotic resistance and biofilm development, it is clear that this research into DSF-like molecules may be only at an initial stage, with future work potentially analyzing the efficacy of these molecules as therapeutic agents.

NUCLEOTIDE-BASED SIGNALS: THE SECONDARY MESSENGERS

cAMP

In recent years, it has become evident that in addition to diffusible communication signals, a large group of nucleotide-based molecules plays a crucial role in controlling bacterial physiology. The first of these signals to be identified in prokaryotes was cyclic AMP (cAMP), reported for the bacterium *Brevibacterium liquefaciens* (later reclassified as *Arthrobacter nicotianae*) in the early 1960s (148). Now recognized as an extensively distributed molecule in bacteria, cAMP is produced by adenylate cyclase enzymes and binds and activates transcription factors from the CRP family (cAMP regulator proteins) (80). In *P. aeruginosa*, synthesis of cAMP is driven primarily by the adenylate cyclases CyaB (PA3217) and, to a lesser extent, CyaA (PA5272) (209). cAMP binding to the CRP-homologous regulator VfR (virulence factor regulator [PA0652]) directly and indirectly controls the production of multiple virulence factors, upregulating exotoxin A, type four pili (TFP), the T3SS, and the Las QS system (1, 10) and downregulating flagellar gene expression (40). Modulation of cAMP levels occurs via the Chp (chemotaxis-like chemosensory system) gene cluster in *P. aeruginosa*, where PilG (PA0408), PilI (PA0410), PilJ (PA0411), ChpA (PA0413), ChpC (PA0415), FimL (PA1822), and FimV (PA3115) upregulate and PilH (PA0409), PilK (PA0412), and ChpB (PA0414) downregulate cAMP levels, establishing a link between Chp and TFP (66, 96). Additionally, mutations in *mucA* (PA0763) and consequent activation of the AlgU (PA0762) regulon have been reported to inhibit cAMP-VfR signaling (97), demonstrating that cAMP-VfR signaling constitutes a complex signaling cascade with multiple regulatory inputs. Infection studies using a mouse model of acute pneumonia with *vfr*, *cyaA*, and *cyaB* mutants revealed a dominant role of CyaB and VfR during infection (184). In parallel to CyaA and CyaB, a third adenylate cyclase with an intriguing mode of action is produced by *P. aeruginosa*. Exoenzyme Y (ExoY [PA2191]) is produced by *P. aeruginosa* and delivered directly via the T3SS to host cells, where it modulates cAMP activity contributing to bacterial virulence (38, 94, 175, 212).

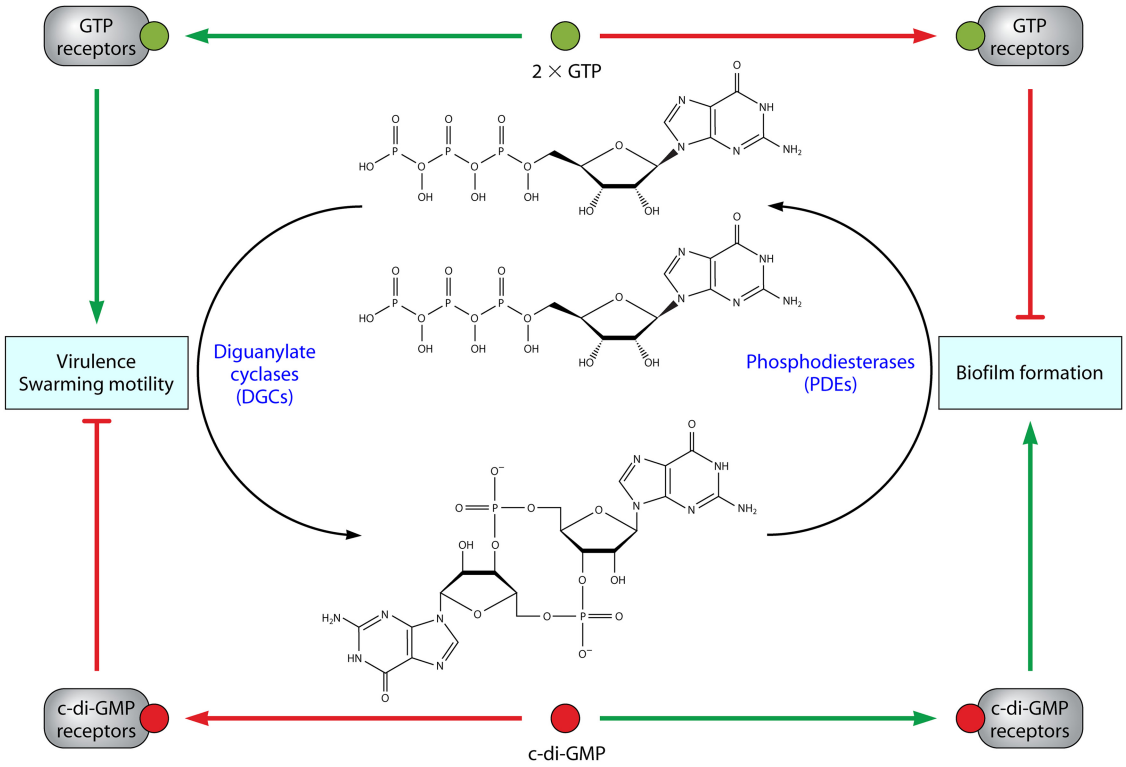


FIG 9 c-di-GMP signaling mechanism. Diguanylate cyclases and phosphodiesterases regulate the bacterial lifestyle (free-living versus biofilm) by balancing the internal levels of c-di-GMP. Binding of c-di-GMP to its receptor targets stimulates biofilm formation, suppressing motility. In parallel, binding of GTP to its receptors (i.e., the allosteric site of the PDE FimX) increases the c-di-GMP-degrading activity of PDE, decreasing c-di-GMP levels, suppressing biofilm formation, and increasing motility.

ppGpp and pppGpp

The second group of nucleotide-based molecules was discovered in 1970 (27) and comprises the cellular alarmones ppGpp and pppGpp. Under amino acid starvation conditions, these molecules rapidly accumulate intracellularly, triggering a switch from cell growth to survival adaptation. In *P. aeruginosa*, AlgQ (AlgR2 [PA5255]) positively regulates the production of the nucleoside diphosphate kinase Ndk (PA3807), responsible for the production of these molecules. Deletion of *algQ* leads to cell death at late exponential phase, a clear consequence of the inability of this mutant to adapt from exponential-phase cell growth to survival mode. This phenotype was rescued by overexpressing AlgQ or Ndk, confirming the role of these proteins in survival adaptation (107).

c-di-GMP

Another nucleotide-based molecule, cyclic di-GMP (c-di-GMP), has attracted even more attention due to its major role as a secondary signaling molecule in many species from all kingdoms. In bacteria, c-di-GMP levels are fine-tuned by the actions of two types of enzymes: diguanylate cyclases (DGCs) containing GGDEF domains, responsible for c-di-GMP synthesis, and phosphodiesterases (PDEs) that contain EAL domains, involved in c-di-GMP degradation (Fig. 9). Together, these enzymes control a multitude of phenotypes in multiple organisms, including biosynthesis of adhesins and exopolysaccharides, motility, long-term survival and environmental stress adaptation, synthesis of secondary metabolites, regulated proteolysis and cell cycle progression, and virulence in plant and animal pathogens.

In addition to enzymes containing single c-di-GMP domains, several proteins contain both GGDEF and EAL domains. These proteins are thus capable of both synthesis and degradation of c-di-GMP, leading to the hypothesis that they act by balancing the internal cellular levels of this molecule. Furthermore, a series of proteins containing degenerate GGDEF domains have been found in multiple organisms. These domains no longer generate c-di-GMP but instead function as allosteric sites (33) or as c-di-GMP receptors (144).

In *P. aeruginosa*, 39 genes have been identified as containing either a DGC, a PDE, or both GGDEF and EAL domains (110). One of the best-studied proteins involved in c-di-GMP formation in this bacterium is the DGC WspR (PA3702). The product of *wspR* is a response regulator linked to the *wsp* operon, which encodes a chemosensory system related to the chemotaxis pathway of *Escherichia coli* (171). Mutations in *wspF* (PA3703), encoding a putative methylesterase related to the chemotactic response protein CheB (177), result in high levels of c-di-GMP and biofilm formation, while a *wspF wspR* double mutant restores the wild-type phenotype. This observation led to the hypothesis that WspF acts by phosphorylating WspR, which stimulates c-di-GMP synthesis (88).

TFP are essential for twitching motility, adherence, and biofilm formation in *P. aeruginosa*. One of the proteins required for TFP formation is FimX (PA4959), a protein essential in twitching mo-

tility (95). FimX contains both an EAL domain and a degenerate GGDEF domain (GDSIF). Biochemical and structural analyses revealed that FimX is capable of degrading but not synthesizing c-di-GMP. The degenerate GGDEF domain exerts an allosteric role, binding c-di-GMP with a high affinity and thereby controlling FimX function (142). FimX is localized at a single cell pole (95); mutagenesis of the EAL and GGDEF domains has demonstrated that both are essential for directing FimX to its correct subcellular location. Slowly but steadily, the mechanism by which FimX controls motility in this bacterium is being elucidated (106). Surprisingly, the major flagellar regulator in *P. aeruginosa*, FleQ, has also been found to be c-di-GMP responsive, despite the absence of GGDEF, EAL, or any other known degenerate domains (87). This recent finding demonstrates that the relatively young c-di-GMP field is examining an extremely complex regulatory system that has still to yield many exciting discoveries.

Furthermore, the commonly occurring linkage between the different signaling systems in *P. aeruginosa* has once again been demonstrated, this time for c-di-GMP and AHLs. In recently published work, Ueda and Wood demonstrated that the Las system indirectly controls the levels of c-di-GMP through the tyrosine phosphatase TpbA (PA3885). Substrate-bound LasR activates expression of *tpbA*, whose product in turn dephosphorylates and inactivates the GGDEF protein TpbB (PA1120), resulting in reduced levels of c-di-GMP and, consequently, increases in exopolysaccharide (EPS) production and biofilm and pellicle formation and a decrease in swarming motility (192, 193).

c-di-GMP and SCV

A clinically relevant phenotype associated with c-di-GMP levels in *P. aeruginosa* is the occurrence of small-colony variants (SCV), an adaptation morphotype associated with late-stage infections in the lungs, antibiotic resistance, hyperadherence, and high levels of EPS production (45, 81, 161, 186, 201). SCV development has been linked to elevated levels of c-di-GMP, indicating that some of these morphotypes could arise from mutations enhancing the activity of DGCs such as WspR. In a recent work, Jenal and coworkers identified a novel c-di-GMP-related operon, *yfiBNR* (PA1119 to PA1121), involved in the regulation of c-di-GMP levels (124). The operon encodes YfiB (an OmpA-like outer membrane lipoprotein), YfiN (also known as TpbP, a membrane-integral DGC), and YfiR (a small periplasmic protein). The *yfiBNR* genes control the production of EPS by upregulating *pel* and *psl* (PA2231-PA2245) expression, a common phenotype observed in SCV variants. Jenal et al. proposed a model in which YfiR represses the activity of the DGC YfiN, reducing the levels of c-di-GMP. Consistent with this model, a cystic fibrosis SCV isolate reverted to the wild type after overexpression of *yfiR*, indicating that this SCV variant arose as a consequence of a mutation in the *yfi* operon. Despite its lower fitness *in vitro*, this mutant persisted for many weeks in a mouse infection model, demonstrating once again the role that SCV morphotypes play in chronic infections and their linkage to mutations that result in elevated c-di-GMP levels.

TARGETING BACTERIAL SIGNALING

A major goal in the study of bacterial cell-to-cell communication systems is the development of novel and efficient antimicrobial agents capable of disrupting virulence. As the first-discovered and most-characterized pathway, AHLs have been the main target of this research. Multiple approaches have been applied in attempts to develop effective AHL-quenching drugs, including research and development of synthetic and natural AHL mimics, use of enzymes to degrade AHLs, and, more recently, the development of AHL antibodies capable of either sequestering or degrading AHLs.

The role of *P. aeruginosa* in human infections has driven most of the research on quorum quenching toward this pathogen. One of the first groups of natural compounds with AHL-quenching activity to be identified was the furanones, produced by the marine alga *Delisea pulchra* (126), which block quorum sensing in *P. aeruginosa*, among other bacteria (84). This research led to a plethora of studies on the production of chemically synthesized furanones with improved quorum-quenching activity (89, 127). Reports show that some furanones are able to inhibit quorum sensing, facilitating clearance of *P. aeruginosa* in a mouse model of lung infection (210). This observation corroborates those obtained from microarray experiments on the effects of furanones on the AHL quorum-sensing regulon (85). Although their exact mode of action remains uncharacterized, it has been suggested that the effects of furanones are related to a reduction in LuxR concentration (125). In addition, protein modeling studies on LuxR receptors (109) and the LasR receptor protein (15) suggest that furanones bind to LasR in the same position as the lactone ring of 3-oxo-C_{12}-HSL. Several further natural and synthetic molecules structurally similar and dissimilar to AHLs have been reported to interfere with AHL quorum sensing by binding to LuxR-type receptors (11, 75, 138, 167, 181, 182), indicating that small-molecule interference with AHL QS systems is an interesting field with several potential applications.

A second approach to inhibit QS-regulated virulence is the identification and improvement of quorum-quenching enzymes capable of disrupting bacterial communication by degrading bacterial signaling molecules. Three types of AHL-quenching enzymes have been reported to date: lactonases, acylases, and oxidoreductases. The first group of quorum-quenching enzymes was initially identified in *Bacillus* spp. (56) and was subsequently found in the genomes of countless bacteria and eukaryotes (26, 54, 133, 145, 153, 170, 203, 213, 216). AHL lactonases belong to either the metallo-β-lactamase family of proteins (108, 121, 190) or the phosphotriesterase-like family (195). Lactonolysis of the AHL ring leads to an inactive product that no longer activates the AHL QS system, with consequent attenuation of virulence, as observed in various infection models (31, 55, 149, 188).

The second class of quorum-quenching enzymes, AHL acylases, was discovered in *Ralstonia* spp. (120) and belongs to the Ntn hydrolase superfamily (16). AHL acylases degrade the amide bond of AHLs, releasing homoserine lactone and an acyl moiety. A marked difference between AHL acylases and AHL lactonases lies in their specificities toward different AHL substrates, in that acylases act upon a more restricted set of AHLs. A comparison between the AHL-binding mechanisms of the *Bacillus thuringiensis* AHL lactonase (108) and the AHL acylase PvdQ from *P. aeruginosa* (13) provides an explanation for this specificity: the long hydrophobic cavity of PvdQ acts as a major selective determinant of substrate stability and catalysis. The *in vivo* virulence reduction upon expression or addition of AHL acylases has also been proven (120, 136, 150), demonstrating that AHL lactonases and AHL acylases are interesting candidates in the development of novel antimicrobial drugs.

Finally, oxidoreductases constitute the third and last family of AHL-quenching enzymes found to date. The oxidoreductase of

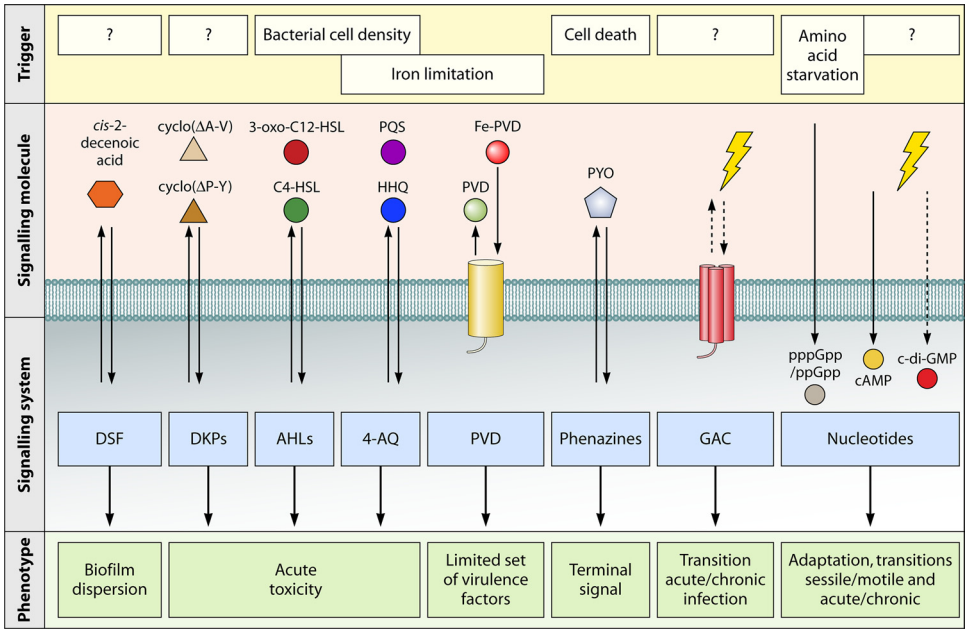


FIG 10 Summary of cell-to-cell signaling systems in *P. aeruginosa*. Dashed lines indicate that the presence of a signal activating the system has been proposed but remains to be proven. The PVD and GAC systems use membrane-associated proteins to activate signaling in response to their respective signals.

Rhodococcus erythropolis W2 has been reported to reduce 3-oxo-substituted AHLs to 3-OH-substituted AHLs (194). In addition, CYP102A1, a cytochrome P450 of *Bacillus megaterium*, was also reported to act as a quorum-quenching enzyme by efficiently oxidizing bacterial AHLs (32). A major drawback that may have limited attention to the study of oxidoreductases as quorum-quenching enzymes resides in the fact that the products still exhibited (markedly reduced) quorum-sensing activity.

A more recent approach to the development of novel antibacterial drugs targeting cell-cell communication is the production of antibodies capable of eliciting an immune response upon detection of the bacterial signal. Binding of antibodies to signaling molecules would interfere with cell-cell communication, resulting in a decrease of virulence. This approach was initially tested in Gram-positive bacteria, with very promising results partially favored by the peptidic nature of their QS signals, which aided in the development of antibodies (4, 152, 214). In order to overcome the small size of AHLs and to potentiate the maximal response for antibody generation, Janda and coworkers (104) pioneered a study where AHLs and AHL analogues were chemically conjugated to keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA), leading to efficient production of AHL-sequestering antibodies. These antibodies have shown very promising efficacy in protecting mice against *P. aeruginosa* infections (103, 104, 135).

The potential for novel antimicrobial drugs targeting 4-quinolone-mediated signaling in *P. aeruginosa* is also being explored. Farnesol, a common sesquiterpene produced by many organisms, was found to inhibit PQS production in *P. aeruginosa*, although the high concentrations required may hamper *in vivo* applications (39). Synthetic anthranilate derivatives (23, 36, 116) have been developed and have shown promising results in a *P. aeruginosa* mouse infection model (116). In parallel, Fetzner and coworkers reported PQS-degrading activity by conversion of PQS to *N*-octanoylanthranilic acid by the dioxygenase Hod (1H-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase) of *Arthrobacter nitroguajacolicus* (162). Hod and the *Pseudomonas putida* homologue QDO (1H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase) (8) belong to an unusual family of cofactor-independent dioxygenases involved in the breakdown of *N*-heteroaromatic compounds (187). A major drawback in the use of Hod against *P. aeruginosa* infections is its sensitivity to degradation by *P. aeruginosa* exoproteases (162), a limiting factor that may be overcome in the following years by protease-resistant variants selected via rational or random mutagenesis.

Besides AHL and 4-AQ systems, little progress has been made in the development of novel drugs targeting the other cell-cell communication systems. The two main explanations for this are that the identities of many signals remain unknown and that little attention has been given to the less-well-studied pathways. To provide some examples, pyoverdine-mediated signaling in *P. aeruginosa* is likely to be disrupted (directly or indirectly) by the addition of pyoverdines from different *Pseudomonas* spp. (22), as well as by the action of molecules that would interfere with the FpvA receptor or proteins that could degrade pyoverdine. In the case of pyocyanin, finding an enzyme capable of efficient degradation of this molecule not only would have consequences on the cell-cell signaling of *P. aeruginosa* but also would have potential biomedical interest due to the cytotoxic effects of pyocyanin (169); however, such approaches have yet to be investigated fully.

CONCLUDING REMARKS

The initial discovery of AHL quorum sensing, followed by the discovery of multiple and different bacterial signaling systems, revolutionized our concept of the control that bacteria have on their behavior in relation to the environment. For decades, bacteria had been considered simple organisms; this early misconception can be attributed to two main misleading observations: first, an underestimation based on the size of these organisms, and

TABLE 1 Interactions between signaling systems in *P. aeruginosa*

Protein	Target system	Effect	Mechanism	Reference(s)
LasR	c-di-GMP	Activation	LasR binding to *tbpA* promoter	192
	PQS	Activation	Binding to *pqsR* promoter	202
PqsE	PYO	Activation	Unknown	52, 71
PvdS	PQS	Activation	Unknown	147
RetS	c-di-GMP	Repression	Unknown	137
RhlR	PQS	Repression	Binding to *pqsR* promoter	202
RsaL	PYO	Repression	Direct mechanism (binding to the *phzA1* and *phzM* promoters) and indirect mechanism (binding to the promoter of LasI and inhibiting its transcription)	42, 163, 165
RsmA	LasI, RhlI	Repression	Binding to *lasI* and *rhlI* promoters	105, 160, 168

second, the apparent absence of communication systems. The term "communication" has largely been associated exclusively with the animal kingdom, whose members are able to transmit messages using verbal or body language. Nonetheless, it now seems obvious that ancient organisms have developed the ability to transfer messages without having to employ sound or movement. Chemistry is unequivocally the most universal cellular language operating in all living organisms; using chemical molecules as signals, all organisms, including bacteria, can produce and detect a large variety of messages that allow individual members to sense and react to the constantly changing environment. By doing so, bacteria have been able to display a dual lifestyle: living as single organisms in the absence of communal obligations and displaying complex social interactions when part of a community. Using chemical signals, bacteria are able to determine population density and diversity, two ecological factors crucial for their survival.

One of the best examples of bacterial adaptation is given by the Gram-negative bacterium *P. aeruginosa.* Initially studied for its implications in human infections, this bacterium soon became a model organism with which to study bacterial signaling due to the high complexity, large degree of adaptability, and rich metabolic diversity that allow its survival in the most hazardous environments, as well as colonization of a large number of hosts. A key factor in the adaptation of *P. aeruginosa* is the large number of signaling proteins encoded in its genome that allow this bacterium to react rapidly to a wide range of signals. In this review, we have selected some of the most clinically relevant examples related to virulence factor production and resistance to conventional antibiotic treatments. The master virulence signaling systems in *P. aeruginosa* are the AHL systems Las and Rhl, which together control the expression of multiple virulence factors in response to cell density. Las and Rhl belong to the Lux-type family of signaling systems, responsible for AHL production, the most extended signals in Gram-negative bacteria. The presence of two AHL signals (3-oxo-C_{12}-HSL and C_4-HSL) in this bacterium is particularly intriguing. Short-chain AHLs are more common among Gram-negative bacteria; therefore, having the Las system (3-oxo-C_{12}-HSL) at the top of the hierarchy may allow *P. aeruginosa* to make its own message prevail over exogenous ones. Another group of signaling molecules related to virulence factor production but encountered lower in the hierarchy are the 4-quinolones. These molecules are highly specific to members of the *Pseudomonas* and *Burkholderia* families. An intriguing fact is that 4-quinolones are strongly upregulated under low-iron conditions, connecting virulence factor production (pyocyanin) with adaptation and survival, an elegant strategy to eliminate competition when survival depends on iron availability.

In parallel to virulence factor production, the second major threat posed by bacterial pathogens is persistence leading to chronic infections, which is linked intrinsically to biofilm formation. In *P. aeruginosa*, the two major systems controlling this transition are the GAC and c-di-GMP systems. Although extracellular signals have not been found for these two systems, their major role in virulence in *P. aeruginosa*, among other bacteria, prompted us to add them to this review. Together, these two signaling cascades play a major role in the switch from highly virulent (acute) to highly persistent (chronic) phenotypes. It is likely that in a versatile bacterium, parallel functions of these systems have been maintained to provide a complex and efficient survival mechanism which can be fine-tuned according to environmental or endogenous signals. Regardless of the importance of the abovementioned signaling systems due to their implications in infections, it remains crucial to maintain efforts in the search and understanding of the complete signaling network of this pathogen (Fig. 10). Some of the signaling systems described here may be considered less relevant due to their limited influence on virulence. However, until we unveil the full network of signals and their corresponding pathways, regulation, and interactions with other systems (Table 1), it is impossible to know the full extent of their involvement in such phenotypes. Thus, each signaling system discovered contributes through its potential as a novel target for antimicrobial drugs. Additionally, understanding the complete signaling integration network of a bacterium may prove essential for determining optimal targets for drug research and discovery.

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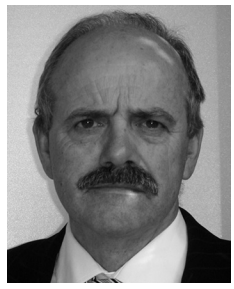

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