Cell-to-Cell Signaling and *Pseudomonas aeruginosa* Infections

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Pseudomonas aeruginosa is a bacterium responsible for severe nosocomial infections, life-threatening infections in immunocompromised persons, and chronic infections in cystic fibrosis patients. The bacterium's virulence depends on a large number of cell-associated and extracellular factors. Cell-to-cell signaling systems control the expression and allow a coordinated, cell-density–dependent production of many extracellular virulence factors. We discuss the possible role of cell-to-cell signaling in the pathogenesis of *P. aeruginosa* infections and present a rationale for targeting cell-to-cell signaling systems in the development of new therapeutic approaches.

Pseudomonas aeruginosa as a Human Pathogen

Pseudomonas aeruginosa, an increasingly prevalent opportunistic human pathogen, is the most common gram-negative bacterium found in nosocomial infections. *P. aeruginosa* is responsible for 16% of nosocomial pneumonia cases (1), 12% of hospital-acquired urinary tract infections (2), 8% of surgical wound infections (3), and 10% of bloodstream infections (4). Immunocompromised patients, such as neutropenic cancer and bone marrow transplant patients, are particularly susceptible to opportunistic infections. In this group of patients, *P. aeruginosa* is responsible for pneumonia and septicemia with attributable deaths reaching 30% (5,6). *P. aeruginosa* is also one of the most common and lethal pathogens responsible for ventilator-associated pneumonia in intubated patients (7), with directly attributable death rates reaching 38% (8). In burn patients, *P. aeruginosa* bacteremia has declined as a result of better wound treatment and dietary changes (removal of raw vegetables, which can be contaminated with *P. aeruginosa*, from the diet) (3). However, *P. aeruginosa* outbreaks in burn units are still associated with high (60%) death rates (9). In the expanding AIDS population, *P. aeruginosa* bacteremia is associated with 50% of deaths (10). Cystic fibrosis (CF) patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population (11). The capacity of *P. aeruginosa* to produce such diverse, often overwhelming infections is due to an arsenal of virulence factors (Figure 1). Many extracellular virulence factors secreted by *P. aeruginosa* have been shown to be controlled by a complex regulatory

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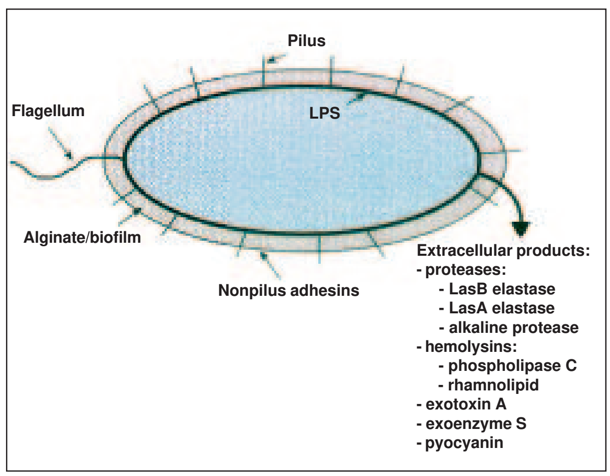

Figure 1. Virulence factors of *Pseudomonas aeruginosa*. *P. aeruginosa* has both cell-associated (flagellum, pilus, nonpilus adhesins, alginate/biofilm, lipopolysaccharide [LPS]) and extracellular virulence factors (proteases, hemolysins, exotoxin A, exoenzyme S, pyocyanin).

circuit involving cell-to-cell signaling systems that allow the bacteria to produce these factors in a coordinated, cell-density–dependent manner (12). In this article we describe major virulence factors of *P. aeruginosa* and the possible involvement of cell-to-cell signaling in the pathogenesis of acute *P. aeruginosa* infection. We also summarize data suggesting that these regulatory systems could be exploited for the design of therapeutic interventions.

Pathogenesis of *P. aeruginosa* Infections

P. aeruginosa (family *Pseudomonadaceae),* an aerobic, motile, gram-negative rod able to grow and survive in almost any environment, lives primarily in water, soil, and vegetation. However, despite abundant opportunities for spread, *P. aeruginosa* rarely causes community-acquired infections in immunocompetent patients. As a result, the pathogen is viewed as opportunistic. The different phases of *P. aeruginosa* infection are shown in Figure 2.

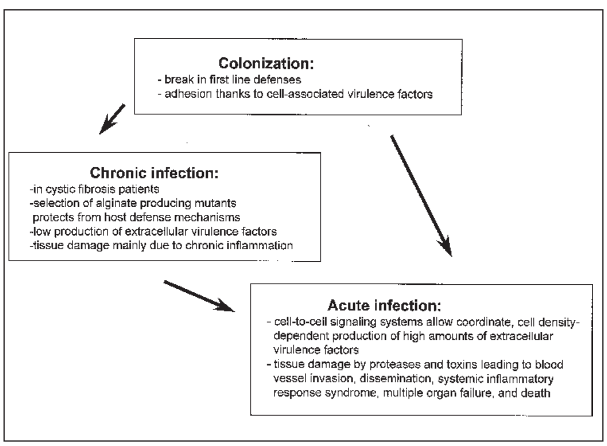

Figure 2. Model of the different phases of *Pseudomonas aeruginosa* infection. After an initial colonization phase, mostly dependent on cell-associated virulence factors, the infectious process evolves either to a chronic infection characterized by low production of extracellular virulence factors or to an acute infection characterized by high production of cell-to-cell signaling dependent virulence factors. During acute exacerbation of chronic infection, the production of cell-to-cell signaling dependent virulence factors is highly increased.

Colonization: The Predominant Role of Cell-Associated Virulence Factors

To initiate infection, *P. aeruginosa* usually requires a substantial break in first-line defenses. Such a break can result from breach or bypass of normal cutaneous or mucosal barriers (e.g., trauma, surgery, serious burns, or indwelling devices), disruption of the protective balance of normal mucosal flora by broad-spectrum antibiotics, or alteration of the immunologic defense mechanisms (e.g., in chemotherapy-induced neutropenia, mucosal clearance defects from cystic fibrosis, AIDS, and diabetes mellitus).

The first step in *P. aeruginosa* infections is colonization of altered epithelium. The pathogen colonizes the oropharynx of up to 6% and is recovered from the feces of 3% to 24% of healthy persons (2). In contrast, up to 50% of hospitalized patients are at high risk for *P. aeruginosa* colonization (2). Adherence of *P. aeruginosa* to epithelium is probably mediated by type 4 pili similar to those of *Neisseria gonorrhoeae* (13). Several other nonpilus adhesins responsible for the binding to mucin have been described, but their role in the infection process remains unclear (14). Flagella, which are primarily responsible for motility, may also act as adhesins to epithelial cells (15).

From Colonization to Acute Infection: The Role of Extracellular Virulence Factors

P. aeruginosa produces several extracellular products that after colonization can cause extensive tissue damage, bloodstream invasion, and dissemination (Figure 1). In vivo studies have shown that mutants defective in the production of exotoxin A, exoenzyme S, elastase, or alkaline protease are essential for maximum virulence of *P. aeruginosa;* however, the relative contribution of a given factor may vary with the type of infection (16). Many of these factors are controlled by regulatory systems involving cell-to-cell signaling. We will summarize the known biologic effects of the most-studied extracellular virulence factors associated with acute *P. aeruginosa* infection.

Exotoxin A is produced by most *P. aeruginosa* strains that cause clinical infections. Like diphtheria toxin, *P. aeruginosa* exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of protein biosynthesis and cell death (17). Exotoxin A is responsible for local tissue damage, bacterial invasion (18), and (possibly) immunosuppression (19). Purified exotoxin A is highly lethal for mice which supports its role as a major systemic virulence factor of *P. aeruginosa* (18).

Exoenzyme S is also an ADP-ribosyl transferase, but unlike exotoxin A, it preferentially ribosylates GTP-binding proteins such as Ras (20). This exoproduct is responsible for direct tissue destruction in lung infection (21) and may be important for bacterial dissemination (22).

Two hemolysins, phospholipase C and rhamnolipid, produced by *P. aeruginosa,* may act synergistically to break down lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects. Rhamnolipid, a rhamnose-containing glycolipid biosurfactant, has a detergentlike structure and is believed to solubilize the phospholipids of lung surfactant, making them more accessible to cleavage by phospholipase C (23). The resulting loss of lung surfactant may be responsible for the atelectasis associated with chronic and acute *P. aeruginosa* lung infection (24). Rhamnolipid also inhibits the mucociliary transport and ciliary function of human respiratory epithelium (25). However, the relative role of rhamnolipid in acute or chronic infection is not known.

Proteases are assumed to play a major role during acute *P. aeruginosa* infection. *P. aeruginosa* produces several proteases including LasB elastase, LasA elastase, and alkaline protease (12). The role of alkaline protease in tissue invasion and systemic infections is unclear; however, its role in corneal infections may be substantial (26,27). The ability of *P. aeruginosa* to destroy the protein elastin is a major virulence determinant during acute infection. Elastin is a major part of human lung tissue and is responsible for lung expansion and contraction. Moreover, elastin is an important component of blood vessels, which rely on it for their resilience. The concerted activity of two enzymes, LasB elastase and LasA elastase, is responsible for elastolytic activity (28). Elastolytic activity is believed to destroy elastin-containing human lung tissue and cause the pulmonary hemorrhages of invasive *P. aeruginosa* infections. LasB elastase is a zinc metalloprotease that acts on a number of proteins including elastin. LasB elastase is highly efficient, with a proteolytic activity approximately 10 times that of *P. aeruginosa* alkaline protease and an activity toward casein approximately four times that of trypsin (28). The LasA elastase is a serine protease that acts synergistically with LasB elastase to degrade elastin. LasA elastase nicks elastin, rendering it sensitive to degradation by other proteases such as LasB elastase, alkaline protease, and neutrophil elastase (28). Both LasB elastase and LasA elastase have been found in the sputum of CF patients during pulmonary exacerbation (29). However, the role of LasB elastase in tissue destruction during the chronic phase of CF is less clear. It has been postulated that during this phase, antibodies present in high titers neutralize LasB elastase, and elastin damaged by minute amounts of LasA is degraded mostly by neutrophil elastase (28). LasB elastase degrades not only elastin but also fibrin and collagen (30). It can inactivate substances such as human immunoglobulins G and A (31), airway lysozyme (32), complement components (33), and substances involved in protecting the respiratory tract against proteases such as α-1-proteinase inhibitor (34) and bronchial mucus proteinase inhibitor (35). Therefore, LasB elastase not only destroys tissue components but also interferes with host defense mechanisms. Studies in animal models show that mutants defective in LasB elastase production are less virulent than their parent strains (16,36,37), which supports the role of LasB elastase as a virulence factor.

Cell-to-Cell Signaling: A Global Regulation System of *P. aeruginosa* Extracellular Virulence Factors

Cell-to-Cell Signaling Systems

P. aeruginosa appears to control the production of many of its extracellular virulence factors by a mechanism that monitors bacterial cell density and allows communication between bacteria by cell-to-cell signaling. Bacteria are able to sense their environment, process information, and react appropriately; however, their ability to sense their own cell density, to communicate with each other, and to behave as a population instead of individual cells has only recently been understood (38,39). This phenomenon, called quorum-sensing or cell-to-cell signaling, is a generic phenomenon described in many gram-negative (40) and gram-positive bacteria (41).

The first cell-to-cell signaling system described is the *lux* system, responsible for the cell-density–dependent control of bioluminescence genes by the marine bacteria *Vibrio fischeri* (38). Cell-to-cell signaling systems of gram-positive bacteria involve peptide pheromones as signals (41). In contrast, cell-to-cell signaling systems of gram-negative bacteria, with the exception of *Ralstonia solanacearum,* in which an endogenous fatty acid derivative has been suggested as a cell-to-cell signal (42,43), are composed of a small molecule called an autoinducer, which is synthesized by a LuxI-type autoinducer synthase and a LuxR-type transcriptional activator protein (R-protein) (Figure 3) (38). The various autoinducers described in gram-negative bacteria are homoserine lactone-based molecules that differ between one another in length and substitutions on their acyl side chains. At low cell density, autoinducer is synthesized at basal levels and is thought to diffuse into the surrounding media,

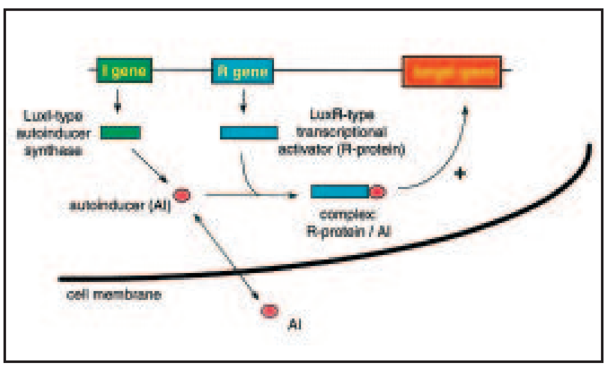

Figure 3. Cell-to-cell signaling systems. Cell-to-cell signaling systems are composed of two genes. The *I* gene encodes an autoinducer synthase and the *R* gene encodes a transcriptional activator protein (R-protein). The autoinducer synthase is responsible for the synthesis of an autoinducer molecule (AI), which crosses the cell membrane. With increasing cell-density the intracellular concentration of AI reaches a threshold level, and the AI then binds to the transcriptional activator. The complex R-protein/AI activates the expression of specific target genes.

where it becomes diluted. With increasing cell density, the intracellular concentration of autoinducer increases until it reaches a threshold concentration. At this critical concentration, the autoinducer has been proposed to bind to a specific R-protein (40). The R-protein itself is not active without the corresponding autoinducer, and it is the R-protein/autoinducer complex that is proposed to bind to specific DNA sequences upstream of target genes enhancing their transcription (44,45). The resulting increase in expression of these genes can reach 1,000-fold. The autoinducer, therefore, allows the bacteria to communicate with each other (cell-to-cell signaling), to sense their own density (quorum-sensing), and together with a transcriptional activator to express specific genes as a population instead of individual cells.

The *las* Cell-to-Cell Signaling System of *P. aeruginosa*

The first cell-to-cell signaling system described in *P. aeruginosa* was shown to regulate expression of LasB elastase and was therefore named the *las* system (46). The *las* cell-to-cell signaling system is composed of *lasI,* the autoinducer synthase gene responsible for the synthesis of 3-oxo-C12-HSL (*N*-[3-oxododecanoyl]-L-homoserine lactone, previously named PAI-1 or OdDHL), and the *lasR* gene that codes for a transcriptional activator protein (Figure 4) (47,48). The *las* cell-to-cell signaling system regulates *lasB* expression and is required for optimal production of other extracellular virulence factors such as LasA protease and exotoxin A (49). *LasI* is the most sensitive gene to activation by LasR/3-oxo-C12-HSL (50). The preference for the *lasI* promoter allows an initial rapid rise in autoinducer synthesis, which increases the amount of 3-oxo-C12-HSL available to bind to LasR. This autoinduction hierarchy is responsible for a dramatic increase of expression of virulence genes (such as *lasB*) once a critical cell density has been reached. Recently, the *las* system has also been shown to activate the *xcpP* and *xcpR* genes that encode proteins of the *P. aeruginosa* secretory pathway (51). 3-oxo-C12-HSL alone has been suggested to contribute to the virulence of *P. aeruginosa* because it has some immunomodulatory activity (52). The *las* cell-to-cell signaling system is positively controlled by GacA (53), as well as by

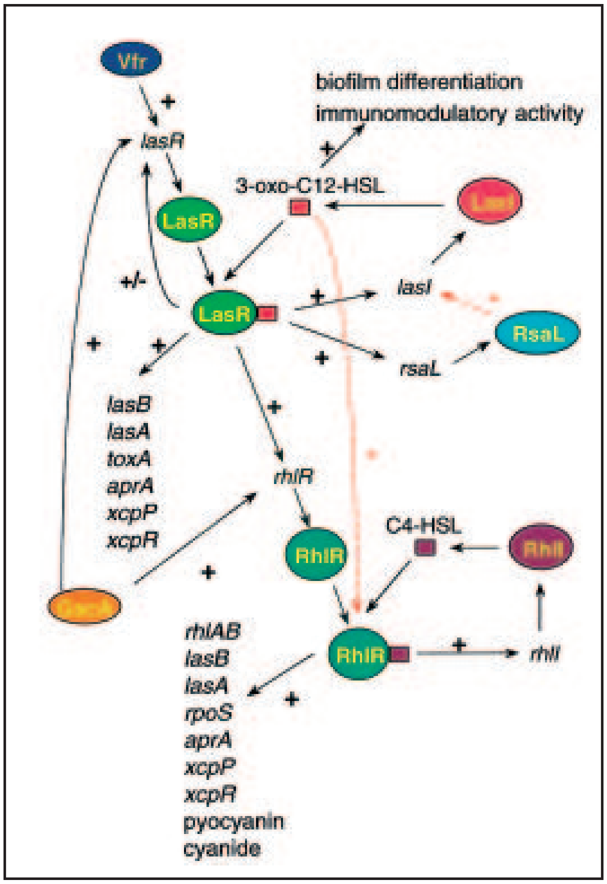

Figure 4. The cell-to-cell signaling circuitry of *P. aeruginosa*. The *las* cell-to-cell signaling system controls the *rhl* cell-to-cell signaling system in a hierarchy cascade. The LasR/3-oxo-C12-HSL complex activates the transcription of *rhlR*, and 3-oxo-C12-HSL blocks the activation of RhlR by C4-HSL. The *las* system itself is controlled positively by Vfr and GacA, and negatively by RsaL. 3-oxo-C12-HSL is required for biofilm differentiation and has immunomodulatory activity. Both cell-to-cell signaling systems regulate the expression of numerous genes (*lasB*: LasB elastase, *lasA*: LasA elastase, *toxA*: exotoxin A, *aprA*: alkaline protease, *xcpP* and *xcpR*: genes of the *xcp* secretory pathway, *rhlAB*: rhamnosyltransferase required for rhamnolipid production, *rpoS*: stationary phase sigma factor).

Vfr, which is required for the transcription of *lasR* (54). An inhibitor, RsaL, that represses the transcription of *lasI,* has also been described (de Kievit et al., unpub. data). The multiple regulatory levels of the *las* cell-to-cell signaling system and the various genes under its control highlight the importance of this system for *P. aeruginosa*.

The *rhl* Cell-to-Cell Signaling System of *P. aeruginosa*

P. aeruginosa has a second cell-to-cell signaling system, named the *rhl* system because of its ability to control the production of rhamnolipid. This system is composed of *rhlI*, the C4-HSL (*N*-butyrylhomoserine lactone, previously named PAI-2 or BHL) autoinducer synthase gene, and the *rhlR* gene encoding a transcriptional activator protein (Figure 4) (55-59). This system regulates the expression of the *rhlAB* operon that encodes a rhamnosyltransferase required for rhamnolipid production (60). The *rhl* system is also necessary for optimal production of LasB elastase, LasA protease, pyocyanin, cyanide, and alkaline protease (53,57,61,62). Therefore, like the *las* cell-to-cell signaling system, the *rhl* system, sometimes referred to as vsm (virulence secondary metabolites), regulates the expression of various extracellular virulence factors of *P. aeruginosa*. Interestingly, the *rhl* system also regulates the expression of *rpoS,* which encodes a stationary sigma factor (δ^S) involved in the regulation of various stress-response genes (63,64).

The Cell-to-Cell Signaling Hierarchy in *P. aeruginosa*

Recent data have shown that the *las* and *rhl* cell-to-cell signaling systems of *P. aeruginosa* interact. Both systems are highly specific in that their respective autoinducers are unable to activate the transcriptional activator protein of the other system (i.e., 3-oxo-C12-HSL cannot activate RhlR, and C4-HSL cannot activate LasR) (57,62). It has also been shown that the R-protein/autoinducer complexes prefer certain promoters that they will activate; LasR/3-oxo-C12-HSL preferentially activates *lasB* over *rhlA*, and RhlR/C4-HSL preferentially activates *rhlA* over *lasB* (57,62). However, neither system is completely independent of the other. The LasR/3-oxo-C12-HSL complex activates the expression of *rhlR* placing the *las* system in a cell-to-cell signaling hierarchy above the *rhl* system (Figure 4) (63,65). Moreover, 3-oxo-C12-HSL can bind to RhlR, blocking the binding of C4-HSL to its transcriptional activator *rhlR* (65). The *las* system therefore controls the *rhl* system at both a transcriptional and posttranslational level. Another yet unidentified regulatory mechanism directly influencing the expression of *rhlRI* has been suggested (63). *Rhl* system regulation of such important genes as *rpoS* could explain why multiple levels of controls are required for its tight regulation.

Cell-to-Cell Signaling: A Powerful System to Overcome Host Defenses during Acute Infection

Cell-to-cell signaling systems might enable *P. aeruginosa* to overcome host defense mechanisms. Isolated production of extracellular virulence factors by a small number of bacteria would probably lead to an efficient host response neutralizing these compounds. However, the coordinated expression of virulence genes by an entire bacterial population once a certain density has been reached might allow *P. aeruginosa* to secrete extracellular factors only when they can be produced at high enough levels to overcome host defenses. These factors could alter the precarious balance between host defenses and production of bacterial toxins, leading to invasion of blood vessels, dissemination, systemic inflammatory-response syndrome, and finally death. Even appropriate antibiotic therapies are often unable to stop this course; therefore, the process must be blocked early, before virulence gene expression can be coordinated.

Biofilms and Cell-to-Cell Signaling

The most striking feature of persistent *P. aeruginosa* infections in CF patients is the selection of mucoid mutants producing the exopolysaccharide alginate (a polymer of mannuronic and guluronic acid) (11). These mutant bacteria grow inside a biofilm (defined as microcolonies surrounded by an exopolysaccharide) as a survival strategy because the surrounding matrix protects bacteria from phagocytes and complement activity (11). *P. aeruginosa* growing in an alginate "slime matrix" are resistant to antibiotics (e.g., aminoglycosides, ß-lactam antibiotics, fluoroquinolones) and disinfectants (11). The exact nature of the increased resistance is unclear but has been attributed to slow growth, penetration barriers, ß-lactamase production, and other factors. *P. aeruginosa* also produces other less well-defined biofilms essential in the colonization of indwelling devices such as catheters. Recently, the *las* cell-to-cell signaling system has been shown to be involved in the differentiation of *P. aeruginosa* biofilms (66). A mutant defective in the production of 3-oxo-C12-HSL formed an abnormal biofilm that, in contrast to the wild type biofilm, was sensitive to low concentrations of the detergent sodium dodecyl sulfate (SDS). Furthermore, the addition of 3-oxo-C12-HSL to the culture media restored production of a differentiated, SDS-resistant biofilm by the mutant (66). Whether the formation of an undifferentiated biofilm renders this mutant more sensitive to antibiotics is still unknown. Also unclear is whether 3-oxo-C12-HSL is required for the differentiation of alginate biofilms. The link between 3-oxo-C12-HSL and biofilm differentiation highlights the broad range of systems controlled by cell-to-cell signaling in *P. aeruginosa*.

Interference with Cell-to-Cell Signaling: A Potential Therapeutic Approach against *P. aeruginosa*

The increasing emergence of bacterial strains resistant to antibacterial drugs is a major challenge. *P. aeruginosa* is one of the most problematic human pathogens, showing intrinsic resistance to many structurally unrelated antibiotics. Resistance mechanisms include low outer membrane permeability or multidrug efflux pumps (tetracycline, imipenem, fluoroquinolones, aminoglycosides) or production of antibiotic modifying enzymes (aminoglycosides, ß-lactams) (67,68). Previous exposure to antibiotics often leads to multidrug-resistant *P. aeruginosa* strains. Moreover, the eradication of colonization with *P. aeruginosa* is almost impossible in CF patients because of the emergence of multidrug-resistant strains and the protective effects of alginate (69). Therefore, major efforts have been made to find new therapeutic interventions against *P. aeruginosa*. A tempting approach is to reduce the production of extracellular virulence factors. In this regard, the interference with cell-to-cell signaling systems is very attractive. Experimental evidence for effective interference with cell-to-cell signaling systems has been recently provided by the use of autoinducer analogs (70-72) or furanone compounds (73). The LasR/3-oxo-C12-HSL complex, which plays a central role in controlling virulence gene expression in *P. aeruginosa,* is essential not only for the production of elastase and other proteases required for tissue invasion but also for the control of the *rhl* cell-to-cell signaling system in a hierarchical cascade and for the differentiation of biofilm. Mutants with a nonfunctional *las* cell-to-cell signaling system are unable to produce several virulence factors (including elastase) and have significantly reduced virulence in a neonatal mouse pneumonia model (37). Therefore, the LasR/3-oxo-C12-HSL complex has become a potential target for new therapeutic interventions. Analogs of 3-oxo-C12-HSL (74) are under investigation for their ability to act as 3-oxo-C12-HSL antagonists, which block activation of LasR and thereby interfere with virulence gene expression. The fact that 3-oxo-C12-HSL can block the bioactivity of C4-HSL (65) suggests that it might be possible to interfere efficiently with cell-to-cell signaling in *P. aeruginosa*. As elements essential for the synthesis of autoinducers have recently been described, drugs inhibiting the biosynthesis process could also be designed (75-80). These approaches could reduce the production of virulence factors, and by interfering with biofilm differentiation they could also render colonizing *P. aeruginosa* more susceptible to antibiotics and biocides. Combination therapies, including antibiotics and drugs reducing virulence factor production, might have an important role in the treatment of CF and intensive-care patients.

As with any therapeutic approach against *P. aeruginosa,* the possible emergence of mutations that would prevent the desired effect is a concern. We have recently described spontaneous mutants that were restored in the production of extracellular virulence factors (elastase and rhamnolipid) in the absence of a functional *las* cell-to-cell signaling system (81). We were unable to isolate mutants restored in virulence factor production when both the *las* and the *rhl* cell-to-cell signaling systems were deficient. These results suggest that therapeutic interventions directed only against the *las* cell-to-cell signaling system are likely to fail because of the emergence of resistant strains able to produce virulence factors despite an inactive *las* system (81). However, if both the *las* and the *rhl* cell-to-cell signaling systems are blocked, *P. aeruginosa* might be unable to restore the production of cell-to-cell signaling dependent virulence factors; this approach may efficiently reduce virulence factor production and high death rates associated with *P. aeruginosa*.

Cell-to-Cell Signaling Systems in Other Human Gram-Negative Pathogens

Other gram-negative human pathogens including *Serratia marcescens*, *S. liquefaciens, Aeromonas hydrophila, Vibrio cholerae, V. parahaemoliticus*, *Yersinia enterocolitica*, *Enterobacter agglomerans, Hafnia alvei*, and *Rahnella aquatis* have been shown to produce homoserine lactone-based autoinducer molecules. These organisms are thought to contain cell-to-cell signaling systems analogous to the *las* and *rhl* systems of *P. aeruginosa* (39,40). *Burkholderia cepacia* (formerly known as *Pseudomonas cepacia*) is an opportunistic pathogen responsible for fatal pulmonary infections in CF patients (11). Virulence factor production of this pathogen can be enhanced by *P. aeruginosa* exoproducts, and this interspecies communication may be due to homoserine-lactone signals (82). Whether *B. cepacia* contains cell-to-cell signaling systems is still unknown. As the number of bacteria known to use cell-to-cell signaling systems increases, therapeutic interference with cell-to-cell signaling might expand to other human pathogens.

Conclusions

The pathogenesis of *P. aeruginosa* is clearly multifactorial as underlined by the large number of virulence factors and the broad spectrum of diseases the bacterium causes. Many of the extracellular virulence factors required for tissue invasion and dissemination are controlled by cell-to-cell signaling systems involving homoserine lactone-based signal molecules and specific transcriptional activator proteins. These regulatory systems enable *P. aeruginosa* to produce virulence factors in a coordinated, cell-density–dependent manner that could allow the bacteria to overwhelm the host defense mechanisms. Interference with cell-to-cell signaling dependent virulence factor production is a promising therapeutic approach for reducing illness and death associated with *P. aeruginosa* colonization and infection. The growing number of human pathogens found to contain cell-to-cell signaling systems highlights the importance of exploring interference with bacterial cell-to-cell signaling for new therapeutic interventions.

Acknowledgment

We thank E.C. Pesci, T. deKievit, and J.P. Pearson for helpful discussions.

Supported by NIH grant R01A133713-04 (to B.H.I.), a Wilmot Foundation, and a Swiss Research Foundation grant (to C.V.D.).

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