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REVIEW

Contribution of an arsenal of virulence factors to pathogenesis of *Pseudomonas aeruginosa* infections

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Abstract *Pseudomonas aeruginosa* is an increasingly prevalent opportunistic pathogen that causes a variety of nosocomial infections, life-threatening diseases in immunocompromised persons and chronic pulmonary infections in cystic fibrosis patients. The organism's virulence depends on an arsenal of cell-associated and extracellular factors determining the pathogenesis of infections as multifactorial. Most *P. aeruginosa* infections are both invasive and toxinogenic. Many of the extracellular virulence factors (proteases, exotoxin A, pyocyanin, siderophores, hemolysins) required for tissue invasion and dissemination of *P. aeruginosa* are controlled by quorum sensing (QS) that enable the bacteria to produce these factors in a coordinated, cell-density-dependent manner and overwhelm the host defense mechanisms during acute infection. Sometimes, QS also contributes to biofilm formation and thus participates in pathogenesis of chronic infection. This system is recognized to be a global regulatory network controlling the expression of a large number of virulence genes either directly or indirectly. Two-component sensor kinases such as RetS, LadS and GacS are also controlling the production of virulence factors as well as the switch from acute to chronic infection. The present review describes the known virulence determinants of *P. aeruginosa*, the stages of infection as well as the importance of QS in the pathogenesis of *P. aeruginosa* infection.

Keywords *Pseudomonas aeruginosa* infections · Pathogenesis · Virulence factors · Quorum sensing

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Introduction

Pseudomonas aeruginosa is an increasingly prevalent opportunistic pathogen that is a common cause of hospital-acquired infections, particularly infecting patients with predisposing factors, such as burn victims, immunocompromised hosts including neutropenic cancer, bone marrow transplant and AIDS patients, or those with metabolic disorders (Pinheiro et al. 2008). In cystic fibrosis (CF) patients, *P. aeruginosa* is believed to be a major contributory factor to chronic lung infections, which could form biofilm and adhere to human mucin in the lower respiratory tract (Whiteley et al. 2001; Finnan et al. 2004). It first colonizes the lungs of children with CF between 5 and 9 years of age and can subsequently be cultured from the sputum of approximately 80% of adults over the age of 18 (Ramsey 1996; Cystic Fibrosis Foundation 2009). The bacterium can only be eradicated in the early stage of colonization, while reduction of bacterial density is desirable during chronic colonization or exacerbations (Cantón et al. 2005). Another group of individuals particularly prone to infections by this organism is hospitalized patients. *Pseudomonas aeruginosa* is responsible for 10–15% of nosocomial infections worldwide (Blanc et al. 1998). It is not surprising that *P. aeruginosa* infections are associated with significant morbidity and mortality due to the organism's capacity to adapt easily to changes in the environment, to rapidly develop resistance to antibiotics, and to produce a variety of virulence factors.

Pseudomonas aeruginosa possesses an arsenal of both cell-associated (flagella, pili, lectins, alginate/biofilm, lipopolysaccharide) and extracellular (proteases, hemolysins, cytotoxin, pyocyanin, siderophores, exotoxin A, exoenzyme S, exoenzyme U, etc.) virulence factors. Production of several *P. aeruginosa* virulence factors is coordinated by a cell

density monitoring mechanism termed Quorum Sensing (QS) (Rumbaugh et al. 2000).

The present review describes the known virulence determinants of *P. aeruginosa*, the stages of infection as well as the involvement of QS (formerly cell-to-cell signaling) in the pathogenesis of *P. aeruginosa* infection.

Pathogenesis of *P. aeruginosa* infections

Pseudomonas aeruginosa is found to grow in almost any environment, primarily in water, soil and vegetation. However, despite abundant opportunities for spread, *P. aeruginosa* rarely causes community-acquired infections in immunocompetent patients. It is an opportunistic pathogen and is only able to infect hosts with defective immune system function. The pathogenesis of *P. aeruginosa* infections is multifactorial, as suggested by the large number and wide array of virulence determinants possessed by the bacterium. Most infections are both invasive and toxinogenic (Todar 2009). The known virulence factors of pathogenic *P. aeruginosa* and their participation in the pathogenesis of infections (as adhesins, invasins, toxins etc.) are presented in Table 1.

The complete *P. aeruginosa* infection is composed of three distinct stages: (1) bacterial adhesion and colonization; (2) local invasion; and (3) disseminated systemic disease. However, the disease process may stop at any stage (Todar 2009). After an initial colonization phase, mostly ensured by cell-associated virulence factors, the infectious process evolves to either an acute infection or a chronic infection. The acute infection is characterized by high production of extracellular virulence factors, regulated by QS, and the chronic infection by low amounts of these determinants (Van Delden and Iglewski 1998). During acute exacerbation of chronic infection, often observed in CF, the production of QS-dependent extracellular virulence determinants is highly increased (Fig. 1).

Table 1 Summary of the known virulence factors of pathogenic *P. aeruginosa*

Pathogenetic function	Cell-associated factors	Extracellular factors
Adhesion	Type IV pili	
	Carbohydrate-binding proteins (lectins)	
	Glycocalix	
	Alginate slime (biofilm)	
Adhesion facilitation		Neuraminidase (sialidase)
Motility/chemotaxis	Flagella (swimming motility)	
	Retractile pili (twitching motility)	
Invasion		Elastases (LasB and LasA)
		Alkaline protease
		Haemolysins (phospholipases and rhamnolipid)
		Cytotoxin (leukocidin)
		Pyocyanin pigment
		Siderophores and siderophore uptake systems
Toxinogenesis	Lipopolysaccharide (endotoxin)	Exotoxin A
	LecA and LecB lectins	Type III effector cytotoxins – ExoS, ExoU, ExoT, ExoY
		Enterotoxin
Dissemination		
Antiphagocytic surface properties	Slime layers	
	Glycocalix	
	Lipopolysaccharide	
Defense against serum bactericidal reaction	Slime layers	Protease enzymes
	Glycocalix	
	Lipopolysaccharide	
Defense against immune responses	Slime layers	Protease enzymes
	Glycocalix	

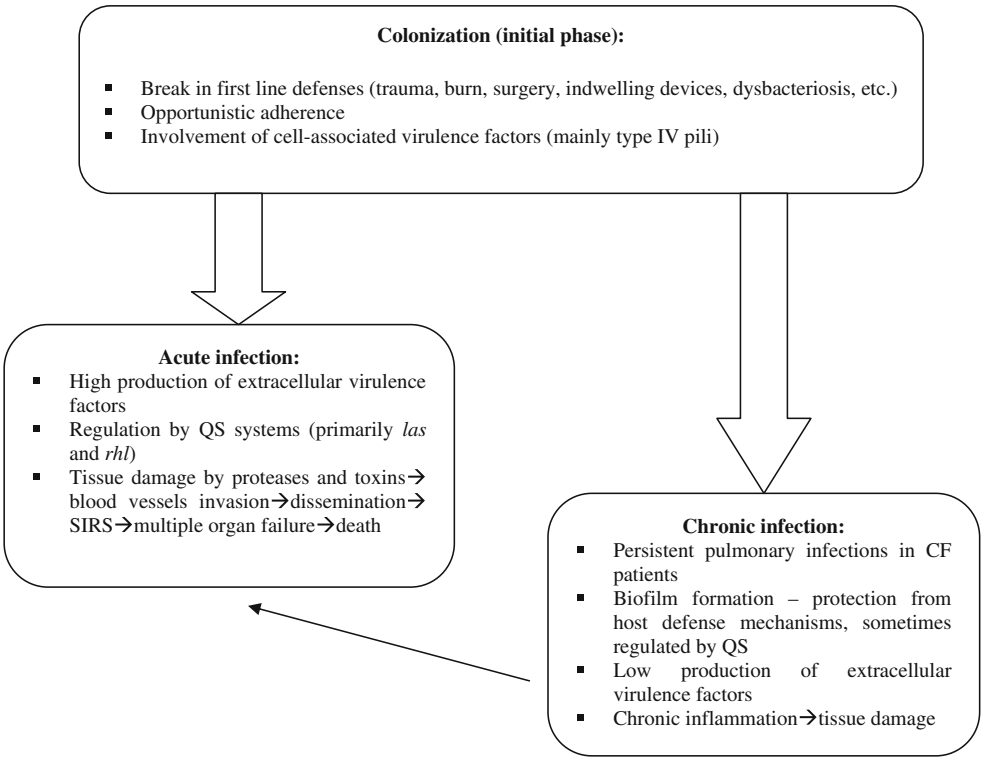

Fig. 1 Phases of *P. aeruginosa* infection: main characteristics. *QS* Quorum sensing, *SIRS* systemic inflammatory response syndrome, *CF* cystic fibrosis

Colonization

Although colonization usually represents the first stage of infections caused by *P. aeruginosa*, the exact source and mode of transmission of the pathogen are often unclear because of its ubiquitous presence in the environment (Todar 2009). Occasionally, *P. aeruginosa* is a part of the normal human flora: it colonizes the oropharynx of up to 6%, and is recovered from the feces of 3–24%, of healthy persons (Pollack 1995). The prevalence of colonization of healthy individuals outside the hospitals is relatively low, whereas up to 50% of hospitalized patients are at high risk for *P. aeruginosa* colonization (Pollack 1995).

The initiation of infection usually requires a substantial break in first-line defenses resulted from breach of normal cutaneous or mucosal barriers (e.g., trauma, severe burns, surgery, or indwelling devices), disruption of the protective balance of normal mucosal flora by broad-spectrum antibiotics and cytostatic drugs, or alteration of the immunologic defense mechanisms (e.g., in chemotherapy-induced neutropenia, AIDS, diabetes mellitus, mucosal clearance defects in cystic fibrosis patients) (Van Delden and Iglewski 1998).

Tissue injury may aid the colonization process of the respiratory tract, since *P. aeruginosa* will adhere to tracheal epithelial cells of mice infected with influenza virus but not to normal tracheal epithelium (Ramphal et al. 1980). This has been called opportunistic adherence, and it may be an important step in *Pseudomonas* keratitis and urinary tract infections, as well as infections of the respiratory tract. Cell-associated virulence factors, mainly pili (also known as fimbriae), participate as adhesins in the initial colonization phase. The pili of *P. aeruginosa* bind to specific galactose or mannose or sialic acid-containing receptors on epithelial cells. Fimbrial adhesion may be facilitated by production of a protease enzyme that degrades fibronectin in order to expose the underlying pilus receptors on the epithelial cell surface (Todar 2009). Recently, it has been shown that the *P. aeruginosa* outer membrane protein OprQ, a member of the OprD superfamily, is involved in the binding of human fibronectin, thereby OprQ plays a possible role in adhesion to epithelial cells, increasing colonization and subsequently enhancing lung destruction by *P. aeruginosa* (Arhin and Boucher 2010).

Polar type IV pili (TFP) are important colonization factors of the opportunistic pathogen *P. aeruginosa* involved in bacteria–host cell interactions (Hahn 1997), in biofilm formation (O'Toole and Kolter 1998) as well as in twitching motility, a unique mechanism of surface propulsion (Semmler et al. 1999; Mattick 2002). Bacteria lacking TFP have been shown to be significantly less able to bind to eukaryotic cells (Farinha et al. 1994) and to be unable to form microcolonies on artificial surfaces, necessary for initiating formation of a biofilm (O'Toole and Kolter 1998). TFP, similar to those of *P. aeruginosa*, are produced by many Gram-negative bacteria including *Neisseria* spp., *Moraxella* spp., *Eikenella* spp., *Dichelobacter* spp. and *Myxococcus* spp. (Strom and Lory 1993). The pilin structural

subunits are produced as prepilins which are cleaved near the N-terminus by a specific prepilin peptidase (PilD/XcpA in *P. aeruginosa*) and N-methylated prior to assembly into the mature pilus fiber (Strom et al. 1993; Lu et al. 1997). As previously described in several studies (Castric and Deal 1994; Spangenberg et al. 1995), the N-terminal amino acid region of mature pilin of *P. aeruginosa* is highly conserved. In contrast, the C-terminus is less conserved and contains a disulfide-bonded loop (DSL) structure. *Pseudomonas aeruginosa* TFP are thought to interact with the eukaryotic glycolipid receptor asialoGM1 (Saiman and Prince 1993; Bryan et al. 1998) via the DSL.

Pseudomonas aeruginosa produces a neuraminidase (sialidase), an enzyme able to cleave α2,3-linked sialic acids (N-acetylneuraminic acids) from sialylated gangliosides (Vimr et al. 2004), thus increasing the amount of asialoGM1, a major receptor for adherence to the respiratory tract (de Bentzmann et al. 1996). Especially, asialoGM1 have been detected in increased amount on the surface of CF respiratory epithelial cells (Saiman and Prince 1993). A potential role for bacterial neuraminidases would be to expose sequestered binding sites within the usually sialylated membrane glycolipids and facilitate attachment to the epithelial surface (Davies et al. 1999). Also, the *P. aeruginosa* neuraminidase is involved in biofilm formation contributing to initial colonization of the airway (Soong et al. 2006). Neuraminidase production has been found to be increased under hyperosmolar conditions present in CF respiratory tract (Cacalano et al. 1992). Moreover, the enzyme production is regulated by genes involved in alginate expression (Cacalano et al. 1992). *Pseudomonas aeruginosa* was first noted to produce neuraminidase by Shilo (Shilo 1957). Three decades ago, Leprat and Michel-Briand further characterized the extracellular neuraminidase produced by a clinical strain of *P. aeruginosa* isolated from a child with CF (Leprat and Michel-Briand 1980). Several recent studies have ascertained that the prevalence of neuraminidase-encoding gene (*nan1*) increases as the clinical state of CF patients worsens (Lanotte et al. 2004; Strateva et al. 2009). These data suggest a key role for the enzyme in CF pulmonary disease evolution.

Flagella, which are primarily responsible for motility (swimming motility), may also act as adhesins to epithelial cells (Ramphal et al. 1996; Feldman et al. 1998). It has been described that flagellin is an adhesin of *P. aeruginosa* responsible for its binding to secreted respiratory Muc1 mucin on the epithelial cell surface (Lillehoj et al. 2001; Lillehoj et al. 2002).

Mucoid strains of *P. aeruginosa*, which produce an exopolysaccharide (alginate slime), have an additional or alternative adhesin which attaches to the tracheobronchial mucin. Alginate slime is a repeating polymer of mannuronic and glucuronic acid that forms the matrix of the biofilm (Sherbrock-Cox et al. 1984). Biofilm formation can take place on a variety of surfaces, such as medical instruments, leading to many types of nosocomial infections, and *P. aeruginosa* has been shown to persist in biofilm in the lungs of CF patients (Costerton et al. 1999; Singh et al. 2000). Biofilms are characterized by a complex, highly structured, bacterial organization (Costerton et al. 1999). They are initiated by the attachment of a single cell on a surface. Multiplication and the development of microcolonies separated by water-filled channels follow this event (Costerton et al. 1995). The *Pseudomonas* biofilm effectively protects the bacteria from the host defenses such as lymphocytes, phagocytosis, the ciliary action of the respiratory tract, opsonization by antibodies and complement deposition (Leid et al. 2005). The ability to form biofilm endows the slime-producing strains with a marked increase in resistance to antibiotics and disinfectants (Costerton et al. 1995). Mah et al. (2003) established that biofilms themselves are not simply a diffusion barrier to the antibiotics, but rather that bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents. They reported a genetic determinant (*ndvB* locus) of the high-level resistance in biofilm-forming *P. aeruginosa*. This locus is required for the synthesis of periplasmic glucans which interact physically with antibiotics and may prevent them from reaching their sites of action by sequestering these antimicrobials in the periplasm. Recently, Zhang and Mah (2008) identified a novel efflux pump in *P. aeruginosa* that is important for biofilm-specific resistance to a subset of antibiotics including aminoglycosides and ciprofloxacin.

Lectins are sugar-binding proteins that bind to carbohydrates located on host cell surfaces and possibly to secreted glycoproteins. *P. aeruginosa* synthesizes two soluble lectins termed LecA and LecB (formerly, respectively named PA-IL and PA-IIL) (Gilboa-Garber 1982). LecA and LecB, specifically binding galactose and fucose, respectively, were initially identified and characterized in the cytoplasm of *P. aeruginosa* (Glick and Garber 1983). Also, large quantities of both of these proteins are present on the outer membrane of the bacteria (Tielker et al. 2005). The *P. aeruginosa* lectins appear to function as adhesins (Wentworth et al. 1991) as well as cytotoxins for respiratory epithelial cells (Chemani et al. 2009). In addition, LecA facilitates the adherence of *P. aeruginosa* to the intestinal epithelium and causes a major defect in the intestinal epithelial barrier, resulting in increased absorption of exotoxin A, an important extracellular virulence factor (Laughlin et al. 2000). Relationships between lectins and other virulence factors of *P. aeruginosa* have been shown; for example, lectin contributions to biofilm formation (Tielker et al. 2005; Diggle et al. 2006a, b), LecB involvement in pilus biogenesis, and protease IV activity (Sonawane et al. 2006).

Invasion

The ability of *P. aeruginosa* to invade tissues depends upon production of extracellular enzymes and toxins that break down physical barriers and damage host cells, as well as resistance to phagocytosis and the host immune defenses (Todar 2009). The bacterium produces several extracellular proteases including LasA elastase, LasB elastase and alkaline protease. These enzymes are assumed to play a major role during acute *P. aeruginosa* infection (Van Delden and Iglewski 1998). The participation of alkaline protease in tissue invasion and systemic infections is unclear; however, its role in corneal infections may be substantial (Howe and Iglewski 1984; Twining et al. 1993; Kernacki et al. 1995). The ability of *P. aeruginosa* to destroy the protein elastin is a main virulence determinant during acute infection. Elastin is an important part of human lung tissue as well as blood vessels and is responsible for lung expansion and contraction. Two enzymes, LasB elastase and LasA elastase, have elastolytic activity (Galloway 1991). Elastases destroy elastin-containing human lung tissue and cause the pulmonary hemorrhages of invasive *P. aeruginosa* infections (Van Delden and Iglewski 1998). Moreover, they degrade both surfactant proteins, SP-A and SP-D, involved in lung innate immunity (Mariencheck et al. 2003; Alcorn and Wright 2004). Several in vitro studies have demonstrated that SP-A and SP-D stimulate alveolar macrophage phagocytosis of *P. aeruginosa* by both opsonic and nonopsonic mechanisms (Manz-Keinke et al. 1992; Mariencheck et al. 1999; Restrepo et al. 1999). The degradation of surfactant proteins with elastase results in inhibition of surface tension-reducing activity as well as in impaired host defense in the lung (Mariencheck et al. 2003).

LasB elastase is a metal chelator-sensitive neutral protease containing zinc that acts on a number of proteins including elastin, fibrin, fibronectin and collagen (Heck et al. 1986; Van Delden and Iglewski 1998). It is highly efficient, with a proteolytic activity approximately 10 times that of *P. aeruginosa* alkaline protease (Galloway 1991). LasA elastase is a serine protease that enhances the activity of LasB by cleaving the glycine–glycine bonds within elastin, rendering it sensitive to degradation by other proteases such as LasB elastase, alkaline protease and neutrophil elastase (Galloway 1991; Kessler et al. 1993). Both elastases, LasB and LasA, have been found in the sputum of CF patients during acute pulmonary exacerbation (Jaffar-Bandjee et al. 1995). However, the role of LasB elastase in tissue destruction during the chronic phase of CF is less clear because antibodies present in high titers neutralize the enzyme. Minute amounts of LasA has been found during this phase and elasin is degraded mostly by neutrophil elastase (Galloway 1991). LasB elastase can inactivate substances such as human immunoglobulins G and A (Heck et al. 1990), airway lysozyme (Jacquot et al. 1985), complement components (Hong and Ghebrehiwet 1992), and substances protecting the respiratory tract against proteases like alpha-1-proteinase inhibitor (Morihara et al. 1979) and bronchial mucus proteinase inhibitor (Johnson et al. 1982). It also acts synergistically with alkaline protease to inactivate the human cytokines gamma-interferon and tumor necrosis factor alpha (Parmely et al. 1990). Therefore, LasB elastase produced by *P. aeruginosa* not only destroys tissue components but also interferes with host defense mechanisms.

Pseudomonas aeruginosa produces three other soluble proteins involved in invasion: a cytotoxin (mw 25 kDa) and two hemolysins. These exoproducts contribute to invasion through their cytotoxic effects on neutrophils, lymphocytes and other eukaryotic cells. The cytotoxin, previously named leukocidin, has been isolated from autolysates of *P. aeruginosa* cells and appears to be associated with all isolates of the species (Baitch et al. 1987). It inactivates eukaryotic cells by forming lesions or pores in the membrane of target cells of the immune system (Lutz et al. 1987). The consequence is increased plasma membrane permeability to small molecules and ions. Such intoxication has been documented in granulocytes (Baltch et al. 1985), endothelial cells (Suttorp et al. 1985), Ehrlich ascites tumor cells (Lutz et al. 1987), and human leukemic cells (Sasak and Lutz 1985). In the case of granulocytes, treatment with the cytotoxin causes an inhibition of the ability of granulocytes to kill *P. aeruginosa* cells (Baltch et al. 1985). In addition, *P. aeruginosa* cytotoxin is localized in the periplasm and has the potential to inhibit macrophage-mediated phagocytosis, possibly by perturbing ion gradients across the macrophage plasma membrane (Kluftinger et al. 1987).

Two hemolysins, hemolytic phospholipase C (PLC-H) and rhamnolipid, appear to act synergistically to break down lipids and lecithin (Van Delden and Iglewski 1998). Heat-stabile rhamnolipid, a rhamnose-containing glycolipid biosurfactant, has a detergent-like structure and is considered to solubilize the phospholipids of lung surfactant, making them more accessible to cleavage by PLC-H (Liu 1974). The resulting loss of lung surfactant may be responsible for the atelectasis observed in both acute and chronic *P. aeruginosa* lung infection (Liu 1979). Rhamnolipid also inhibits the muco-ciliary transport and ciliary function of human respiratory epithelium (Read et al. 1992).

One of the substrates for PLC-H is phosphatidylcholine, which is hydrolyzed to release phosphorylcholine and diacylglycerol. PLC-H preferentially hydrolyzes phospholipids containing quaternary ammonium groups, which are found primarily in eukaryotic membranes and lung surfactant (e.g., phosphatidylcholine), but has little activity toward phospholipids found in the prokaryotic membrane (e.g., phosphatidylethanolamine) (Berka and Vasil 1982).

Pseudomonas aeruginosa also produces a nonhemolytic phospholipase C (PLC-N) which is 40% identical to the PLC-H (Ostroff et al. 1990). It is nonhemolytic for human or sheep erythrocytes. Both phospholipases hydrolyze phosphatidylcholine; however, each enzyme has a distinct substrate specificity. PLC-H hydrolyzes sphingomyelin in addition to phosphatidylcholine, whereas PLC-N is active on phosphatidylserine as well as phosphatidylcholine (Ostroff et al. 1990).

One *P. aeruginosa* pigment is probably involved in the invasion stage of infection. Pyocyanin (PCN), a blue redox-active secondary metabolite, is a member of a large family of tricyclic compounds known as phenazines (Ran et al. 2003). The presence of high concentrations of PCN in the sputa of CF patients has suggested that this compound plays a role in pulmonary tissue damage observed with chronic lung infection (Wilson et al. 1988). In vitro investigations have revealed that PCN interferes with a large number of mammalian cell functions including cell respiration, ciliary beating, epidermal cell growth (Wilson et al. 1988), calcium homeostasis (Denning et al. 1998), prostacyclin release from lung endothelial cells (Kamath et al. 1995), apoptosis in neutrophils (Usher et al. 2002), release of interleukin-2 (limits growth of T-lymphocytes), secretion of immunoglobulins by B-lymphocytes (Mühlradt et al. 1986), and imbalance of protease-antiprotease activity in the airways of CF patients (Britigan et al. 1999).

A derivative of pyocyanin, pyochelin, is a siderophore that is produced under low-iron conditions to sequester iron from the environment for growth of the pathogen (Cox 1980). It could play a role in invasion if it extracts iron from the host to permit bacterial growth in a relatively iron-limited environment.

Dissemination

Bloodstream invasion and dissemination of *P. aeruginosa* from local sites of infection is mediated by the same cell-associated and extracellular virulence factors responsible for the localized disease, although it is not quite clear how the bacterium causes a systemic illness. *Pseudomonas aeruginosa* is resistant to phagocytosis and the serum bactericidal response due to its slime layer and possibly lipopolysaccharide (LPS). The proteases inactivate complement components (Hong and Ghebrehiwet 1992), IgG and IgA antibodies (Heck et al. 1990), and cytokines such as gamma-interferon and tumor necrosis factor alpha (Parmely et al. 1990). The lipid A moiety of *Pseudomonas* LPS (endotoxin) mediates the usual pathologic aspects of Gram-negative septicemia (e.g., fever, hypotension, disseminated intravascular coagulation, etc.). It is also assumed that *P. aeruginosa* Exotoxin A facilitates the dissemination of infection. Increased titers of anti-exotoxin A antibodies in serum from patients with *P. aeruginosa* sepsis have shown to be associated with better survival in some studies (Kurahashi et al. 1999).

Toxinogenesis

Pseudomonas aeruginosa produces several different ADP-ribosyltransferase toxins (Woods and Iglewski 1983). Exotoxin A (ETA) is produced by the majority of *P. aeruginosa* strains that cause clinical infections (Van Delden and Iglewski 1998). Similarly to diphtheria toxin, it catalyzes ADP-ribosylation and inactivation of elongation factor 2, leading to inhibition of eukaryotic protein biosynthesis and cell death (Wick et al. 1990). ETA consists of two subunits: fragment A is catalytic, and fragment B is responsible for interaction with eukaryotic cell receptors (Woods and Iglewski 1983). It utilizes a different receptor on host cells than the exotoxin produced by *Corynebacterium diphtheriae*, but otherwise enters cells in the same manner and has the exact enzymatic mechanism. ETA is highly toxic to numerous mammalian cells (Middlebrook and Dorland 1977), stimulates in vitro production of interleukin-1 in murine peritoneal macrophages (Misfeldt et al. 1990), and induces murine cytotoxic T-lymphocytes (Zehavi-Wilner 1988). This extracellular virulence factor appears to mediate both local and systemic disease processes caused by *P. aeruginosa* (Kurahashi et al. 1999). ETA has necrotizing activity at the site of bacterial colonization and is thereby thought to contribute to the colonization process. Also, it is responsible for bacterial invasion (Woods and Iglewski 1983), and immunosuppression (Vidal et al. 1993).

An important and recently recognized virulence determinant of *P. aeruginosa* is the complex type III secretion system which injects effector proteins into host cells (Hauser 2009). The genes encoding the secretion, translocation and regulatory machinery of this system are clustered together in the 55-min region of the *P. aeruginosa* chromosome (http://www.pseudomonas.com). In contrast to the clustered genes encoding the type III transport machinery, the genes encoding the type III effector proteins appear to be scattered throughout the chromosome (Stover et al. 2000). Moreover, the genes encoding some *P. aeruginosa* type III effector proteins are variable traits (i.e. they are found in some isolates but not in others) (Feltman et al. 2001; Strateva et al. 2010). To date, four effector proteins secreted by a type III secretion pathway, have been identified in *P. aeruginosa*: exoenzyme S (ExoS), exoenzyme U (ExoU), exoenzyme T (ExoT) and exoenzyme Y (ExoY) (Engel and Balachandran 2009).

ExoS and ExoT are closely related bifunctional proteins (74% identity at the amino acid level) (Yahr et al. 1996a).

ExoS has been more extensively studied. Its N-terminal domain possesses an arginine finger motif characteristic of GTPase activating proteins (GAPs). ExoS manifests GAP activity towards Rho, Rac, and Cdc42 in vitro and in vivo and has been shown to be sufficient to disrupt the actin cytoskeleton (Goehring et al. 1999; Pederson et al. 1999; Krall et al. 2002). The C-terminal domain of ExoS reveals ADP-ribosyltransferase (ADPRT) activity towards Ras, Ral, and various Rab family GTPases and requires a eukaryotic 14-3-3 protein for activity (Ganesan et al. 1998; Zhang et al. 1999; Barbieri et al. 2001). This activity interferes with eukaryotic DNA synthesis and endocytosis and causes cytotoxicity and cell death of mammalian cells (Barbieri et al. 2001; Pederson and Barbieri 1998). ExoS is directly translocated into eukaryotic cells by the contact-dependent type III secretory process (Yahr et al. 1996b). Bacterial translocation of ExoS into epithelial cells results in a general inactivation of cellular function, as recognized by the inhibition of DNA synthesis, loss of focal adhesion, cell rounding, and microvillus effacement (Olson et al. 1999). This exoproduct is responsible for direct tissue destruction in lung infection (Nicas et al. 1985b) and may be important for bacterial dissemination (Nicas et al. 1985a).

The prevalence of ExoT-producing clinical strains of *P. aeruginosa* is higher than that of ExoS-producing strains (Feltman et al. 2001). ExoT is also an ADP-ribosyltransferase but has only 0.2% of the catalytic activity of ExoS (Yahr et al. 1996a). Like ExoS, it has N-terminal GAP activity in vitro and in vivo for Rho, Rac, and Cdc42 (Krall et al. 2000; Kazmierczak and Engel 2002). This activity contributes to disruption of the actin cytoskeleton, resulting in cell rounding (but not cytotoxicity), prevention of bacterial internalization by epithelial cells and macrophages (Cowell et al. 2000; Garrity-Ryan et al. 2000), and inhibition of wound healing (Geiser et al. 2001). In addition, the ADPRT domain of ExoT is functional in vivo and acts synergistically with the GAP activity to disrupt the actin cytoskeleton (Garrity-Ryan et al. 2004).

Of the type III effector proteins, ExoU (also called PepA) (mw 74 kDa) is the most cytotoxic (Finck-Barbancon et al. 1997; Hauser et al. 1998). It acts as an acute cytotoxin in vitro and is associated with accelerated lung injury in vivo (Finck-Barbancon et al. 1997). ExoU is found in about one-third of clinical isolates (Feltman et al. 2001), and these ExoU-producing strains are associated in 90% of cases with severe disease (Hauser et al. 2002; Schulert et al. 2003). It is implicated along with ExoT in the onset of septic shock (Kurahashi et al. 1999) and possesses in vivo phospholipase A2 activity (Phillips et al. 2003). Sepsis appears to arise from epithelial cell damage and subsequent leakage of proinflammatory cytokines into the bloodstream, giving rise to a systemic inflammatory response. The combined actions of ExoU as a potent cytotoxin and ExoT in its ability to inhibit bacterial internalization and wound repair (Garrity-Ryan et al. 2000; Geiser et al. 2001) are also seen to be important in a murine model of acute pneumonia (Allewelt et al. 2000). ExoU leads to rapid cytolytic cell death of a variety of mammalian cell types in vitro, including macrophages, epithelial cells, CHO cells, and fibroblasts (Finck-Barbancon et al. 1997; Vallis et al. 1999; Hauser and Engel 1999). Although the importance of ExoU in infection is well established, the mechanism by which this toxin kills host cells is less clear. Recent study has indicated that a C-terminal domain between residues 550 and 687 of ExoU targets this toxin to the plasma membrane of host cells and the process may be important in cytotoxicity (Rabin and Hauser 2005; Rabin et al. 2006). During or after this localization, ExoU interacts with a host cell factor that activates its phospholipase A2 activity. This activity then causes cell death by utilizing plasma membrane phospholipids as substrates. Death may occur by direct disruption of the plasma membrane integrity or indirectly by perturbation of signaling cascades involving phospholipids, such as the prostaglandin pathway (Rabin et al. 2006).

Most strains of *P. aeruginosa* carry the encoding gene for either ExoS or ExoU but not both (Feltman et al. 2001). The carriage of *exoS* is more prevalent among *P. aeruginosa* isolates. Interestingly, the *exoU* and *exoS* genes do not reside at the same locus (Stover et al. 2000). Several studies have indicated that the determinants for expression and secretion of ExoU (the *exoU* gene and the *spcU* gene encoding its cognate chaperone (Finck-Barbancon et al. 1998)) are located within the small (11-kb) pathogenicity island PAPI-2 of *P. aeruginosa* (He et al. 2004; Kulasekara et al. 2006). Pathogenicity islands are specialized genomic islands that encode virulence determinants. Genomic islands are segments of bacterial genomes that have been acquired through horizontal gene transfer (Qiu et al. 2006). They are often integrated at sites adjacent to tRNA genes, are flanked by direct repeats, greatly differ in their G+C content from those of the core genome, and contain mobile genetic elements (Dobrindt et al. 2004; Schmidt and Hensel 2004). In addition, genomic islands are subject to rearrangements resulting from translocation of a portion of an island to a different location on the chromosome.

The fourth type III effector protein, ExoY, is an adenylate cyclase that elevates the intracellular cAMP levels in eukaryotic cells and causes rounding of certain cell types (Yahr et al. 1998; Vallis et al. 1999). A eukaryotic protein distinct from calmodulin stimulates the adenylate cyclase activity of ExoY. Moreover, the production of ExoY may play a role in the protection of the bacterium from local phagocytic cells.

Strains of *P. aeruginosa* are capable of producing an enterotoxin that is probably responsible for diarrheal diseases (Kubota and Liu 1971; Liu 1974; Shriniwas and Bhujwala 1979).

Protein secretion systems in *P. aeruginosa*: virulence weapons allowing a successful infection of the host

Protein secretion systems in bacterial pathogens are virulence weapons allowing bacteria to successfully infect the host (Merrell and Falkow 2004). Secreted proteins are essential for nutrients or iron acquisition, to avoid the host immune system, to destroy host cell signaling, and to replicate within macrophages; in other words to adapt, survive and exploit the hostile environment of the host. These proteins could remain bound at the bacterial cell surface, be freed up into the extracellular medium or be injected into the host cell cytosol (Bleves et al. 2010). To date, six different classes of secretion systems have been described in Gram-negative bacteria, identified as type I secretion system (T1SS) up to type VI secretion system (T6SS) (Durand et al. 2009). Secretion via T2SS and T5SS is accomplished in a two-step process including a stopover in the periplasm (Michel and Voulhoux 2009), whereas T1SS, T3SS, T4SS and T6SS use a one-step mechanism, promoting the direct delivery of exoproteins into the extracellular medium (T1SS) or into the host cell (T3SS, T4SS and T6SS) (Bleves et al. 2010).

Except for T4SS, all the other secretion machines described in Gram-negative bacteria have been found in *P. aeruginosa* (Bleves et al. 2010). Using this secretion arsenal, *P. aeruginosa* is able to produce and release a large number of exoproteins, including toxins and hydrolytic enzymes.

Type I secretion system (T1SS)

In *P. aeruginosa*, two T1SS have been studied. The Apr system consists of AprD [ABC (ATP-binding cassette) transporter, which is inserted in the inner membrane (IM)], AprF [outer membrane (OM) protein], and AprE (adaptor protein connecting these two components). It is involved in the extracellular secretion of the alkaline protease AprA and of AprX, a protein of unknown function (Guzzo et al. 1991; Duong et al. 2001). The other T1SS is involved in iron utilization. This system consists of HasD (ABC transporter), HasE (adaptor), and HasF (OM protein). The secreted protein HasAp is a hemophore, which binds haem from hemoglobin and is likely a crucial component for *P. aeruginosa* survival in the early stages of infection (Letoffe et al. 1998; Wandersman and Delepelaire 2004).

Type II secretion system (T2SS)

In *P. aeruginosa*, the Xcp (extracellular protein) T2SS is encoded by a set of 11 genes organized in two divergent operons, *xcpP* to *Q* and *xcpR* to *Z*, whereas the 12th gene, *xcpA*/*pilD*, is located outside of the two *xcp* operons. *xcpA*/*pilD* encodes a peptidase which has a role in the maturation of the five T2SS pseudopilins (XcpT-X) and all other known pseudopilins and pilins of *P. aeruginosa* except for the Flp pilin (Ruer et al. 2007). Pseudopilins are first targeted and exported across the IM by the Sec/SRP pathway (Arts et al. 2007). The major pseudopilin XcpT is then assembled into a fimbrial structure called pseudopilus (Durand et al. 2003).

In addition to the initially characterized T2SS Xcp, *P. aeruginosa* possesses a second T2SS only functional in phosphate-limiting growth conditions, the Hxc system (Ball et al. 2002).

Pseudomonas aeruginosa produces and releases a broad variety of T2SS-dependent exoproteins (Bleves et al. 2010) including: proteolytic enzymes such as the elastase LasB, the staphylolysin LasA, the aminopeptidase PaAP and the protease IV; enzymes catalyzing lipid metabolism such as LipA and LipC lipases, as well as phospholipases C; two alkaline phosphatases, PhoA and LapA; one chitin-binding protein called CbpD; and exotoxin A (ToxA).

Type III secretion system (T3SS)

The T3SS is responsible for the delivery of effectors directly into the cytosol of eukaryotic cells (Hauser 2009). The four *P. aeruginosa* T3SS effectors (ExoS, ExoU, ExoT, ExoY) are described in detail above (see "Toxinogenesis").

Type V secretion system (T5SS)

The T5SS is the simplest secretion pathway described so far. Similar to T2SS, T5SS is a two-step process. First, proteins cross the IM via the Sec export machinery; second, they are transported through an OM channel formed by a β barrel protein. Finally, exoproteins either remain associated with the OM or are released into the extracellular medium after a proteolytic cleavage (Bleves et al. 2010). Two subtypes of T5SS exist in Gram-negative bacteria: the autotransporters (AT or T5aSS and T5cSS) and the two-partner secretion (TPS or T5bSS) (Bleves et al. 2010).

EstA is the only characterized AT in *P. aeruginosa* (Wilhelm et al. 1999). It possesses an esterase activity, i.e. EstA can hydrolyze glycerol esters with short- or long-chain fatty acid. Also, EstA is involved in the production of rhamnolipids, and an *estA* defective mutant was shown to be deficient in twitching, swarming and swimming motilities, and thus in biofilm formation (Wilhelm et al. 2007).

T5bSS-dependent exoproteins are: LepA and CupB5. LepA is a protease that activates the critical transcription factor NF-kappaB for host inflammatory and immune responses through digestion of human specific receptors

(Kida et al. 2008). The *cupB5* gene is located within the *cupB* cluster encoding chaperone usher pathway components involved in the assembly of CupB fimbriae (Ruer et al. 2008).

Type VI secretion system (T6SS)

The T6SS was recognized in *P. aeruginosa* a few years ago (Mougous et al. 2006). The exoproteins secreted by this pathway essentially belong to two families, namely Hcp (hemolysin-coregulated protein) and VgrG (valine-glycine repeat) (Filloux 2009; Hachani et al. 2011). Hcp is a small protein which forms nanotubes on the bacterial surface. These tubes may allow transport of other T6SS-dependent effector proteins (Mougous et al. 2006). VgrGs contain two domains, which are related to proteins constituting the bacteriophage tail (Leiman et al. 2009). It has been proposed that the VgrGs form trimeric complexes that could be used as puncturing devices to perforate membranes and allow the passage of proteins and macromolecular complexes (Hachani et al. 2011).

Quorum sensing: a global regulatory network of *P. aeruginosa* extracellular virulence factors

Pseudomonas aeruginosa appears to control the production of several of its extracellular virulence factors by a unique mechanism that monitors bacterial cell density and allows communication between bacteria by intercellular signaling (Van Delden and Iglewski 1998; Smith and Iglewski 2003). Bacteria sense their environment, process information, and react appropriately. Moreover, they are able to sense their own cell density, to communicate with each other, and to behave as a population instead of individual cells (Lazdunski et al. 2004). This mechanism, called quorum sensing (QS), is a generic phenomenon described in many Gram-negative (Whitehead et al. 2001) and Gram-positive bacteria (Kleerebezem et al. 1997).

In common, QS systems of Gram-negative bacteria, including *P. aeruginosa*, are composed of an acyl-homoserine lacton (AHL) signaling molecule called an autoinducer (AI) and a LuxR-type transcriptional activator protein termed R-protein (Fuqua et al. 1996). AI is synthesized by a LuxI-type autoinducer synthase. At low cell density, AI is synthesized at basal levels and is thought to diffuse into the surrounding media, where it becomes diluted. With growth of bacterial population and increasing cell density, the intracellular concentration of AI increases until it reaches a threshold concentration. At this critical concentration, the AI binds to a specific R-protein (Whitehead et al. 2001). The R-protein/AI complex formed is able to bind to specific DNA sequences upstream of target genes enhancing their transcription. Therefore, this regulatory mechanism allows the bacteria to communicate with each other (intercellular signaling), to sense their own density (quorum sensing) and to express specific genes as a population instead of individual cells (Lazdunski et al. 2004).

Currently, *P. aeruginosa* is known to have two primary AHL systems, *las* and *rhl* (Smith and Iglewski 2003). Each system contains homologs of the LuxR and LuxI proteins of the prototypic *lux* QS system from *Vibrio fischeri* (Fuqua et al. 1996). The *las* system consists of the transcriptional activator protein LasR and of LasI, which directs the synthesis of the autoinducer PAI-1 [*N*-(3-oxododecanoyl)-L-homoserine lactone] (Gambello and Iglewski 1991; Pearson et al. 1994). This system has been shown to activate the expression of *lasI*, *lasB*, *lasA*, *apr*, and *toxA* genes (Gambello et al. 1993; Seed et al. 1995; Toder et al. 1991). Similarly, the *rhl* system consists of the transcriptional activator protein RhlR and RhlI, which directs the synthesis of the autoinducer PAI-2 (*N*-butyryl-L-homoserine lactone) (Ochsner and Reiser 1995; Pearson et al. 1995). This system controls the expression of *rhlI* and *rhlAB*, which codes for a rhamnosyltransferase required for rhamnolipid (heat-stabile hemolysin) production (Ochsner et al. 1994; Ochsner and Reiser 1995). It has been shown that the *las* QS system controls the *rhl* QS systems in two ways (Pesci et al. 1997). First, LasR and PAI-1 activate *rhlR* transcription. Second, PAI-1 blocks PAI-2 from binding to RhlR, thereby inhibiting the expression of *rhlA*. Thus, the *las* system exerts two levels of control on RhlR, transcriptional and posttranscriptional (Pesci et al. 1997).

The *rhl* QS system has been reported to activate the transcription of *rpoS* (sigma factor RpoS-encoding gene) (Latifi et al. 1996; Schuster et al. 2004). Vice versa, the stationary sigma factor RpoS was demonstrated to be involved in the modulation of expression of a large number of QS genes (such as *toxA*, *lecA*, *lecB* and pyocyanin-encoding genes) as well as the type III secretion system and anti-host effector proteins (Whiteley et al. 2000; Winzer et al. 2000; Hogardt et al. 2004; Schuster et al. 2004). The two hierarchically arranged *lasR* and *rhl* QS circuits are subject to modulation by a number of additional regulators, which fine-tune the QS response of *P. aeruginosa* (Juhas et al. 2005).

The described QS systems are embedded into a large regulatory network, which includes in particular a third communication molecule, 2-heptyl-3-hydroxy-4-quinolone, more commonly known as the *Pseudomonas* quinolone signal (PQS) (Pesci et al. 1999; Diggle et al. 2006a, b). PQS regulates diverse target genes including those coding for elastase, rhamnolipid and pyocyanin as well as influencing biofilm development and impacting on cellular fitness (Diggle et al. 2006a, b). In addition to PQS, a

precursor of PQS biosynthesis, 4-hydroxy-2-heptylquinoline is also secreted from the cell and may act as a signaling molecule (Deziel et al. 2004).

The QS regulatory network as well as the QS-controlled functions in *P. aeruginosa* are presented in Fig. 2. Several studies have demonstrated that QS controls the expression of at least 400 genes either directly or indirectly (Whiteley et al. 1999; Schuster et al. 2003; Wagner et al. 2003).

Quorum sensing: a powerful system to overwhelm host defenses during acute infection

During acute *P. aeruginosa* infection, the production of many QS-dependent extracellular virulence factors is enough high to overcome host defenses. These factors could alter the precarious balance between host defenses and production of bacterial invasins and toxins (elastases, alkaline protease, pyocyanin, rhamnolipid, exotoxin A, etc.), leading to invasion of blood vessels, dissemination, systemic inflammatory response syndrome, multiple organ failure and finally death (Van Delden and Iglewski 1998).

Involvement of quorum sensing in chronic *P. aeruginosa* infection

Pseudomonas aeruginosa is the predominant microorganism in chronic lung infection of CF patients (Cystic Fibrosis Foundation 2009). The onset of the chronic lung infection is preceded by intermittent colonization usually with environmental strains (Schelstraete et al. 2008). When the chronic infection becomes established, it is well accepted that the isolated *P. aeruginosa* strains differ phenotypically from the intermittent strains. Dominating changes are the switch to mucoidity (alginate overproduction) and loss of epigenetic regulatory systems such as QS (Bjarnsholt et al. 2010). During long term infection of the CF lung, *P. aeruginosa* isolates may lose LasR dependent QS but keep the capability of RhlR-dependent QS regulation enabling production of a number of important host-damaging virulence factors, particularly rhamnolipids which provide a shield against cellular components of the innate immune response (Alhede et al. 2009; Bjarnsholt et al. 2010).

As previously announced, the *las* QS system has been shown to be involved in differentiation of *P. aeruginosa* biofilms (Davies et al. 1998). A mutant defective in the production of *N*-(3-oxododecanoyl)-L-homoserine lactone

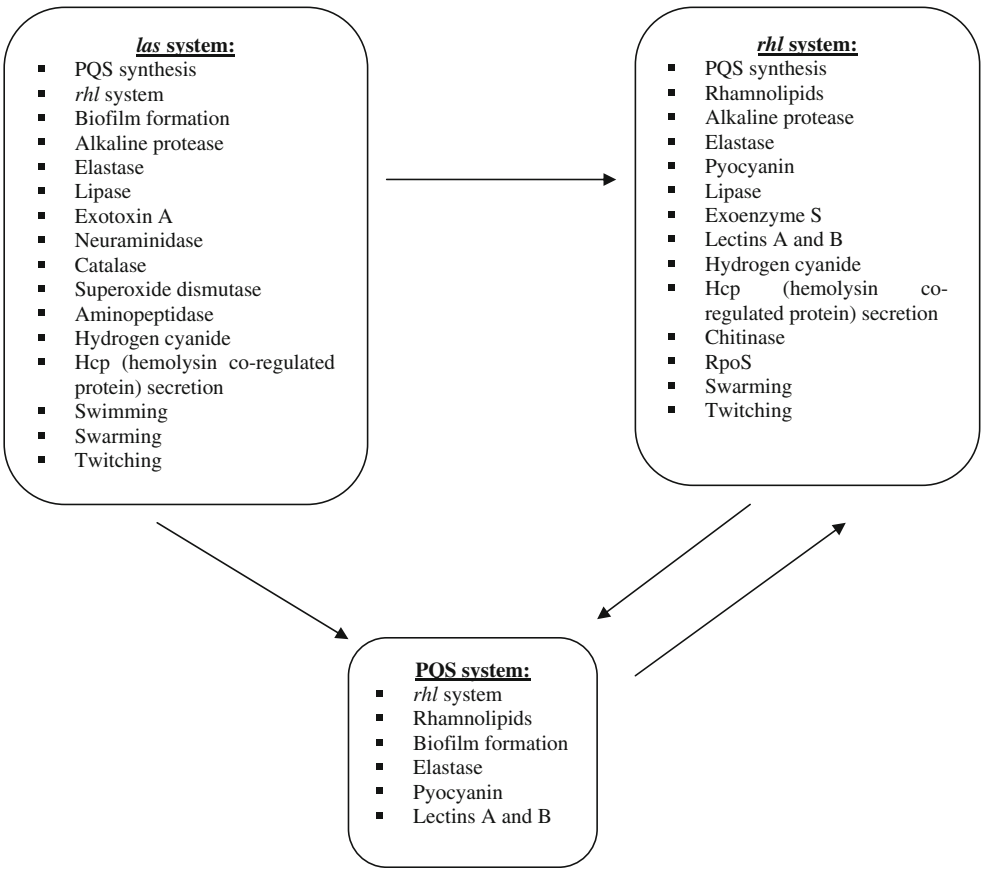

Fig. 2 Functions controlled by the quorum sensing circuitry in *P. aeruginosa*

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 *N*-(3-oxododecanoyl)-L-homoserine lactone to the culture media restored production of a differentiated, SDS-resistant biofilm by the mutant (Davies et al. 1998). Smith et al. (2006) demonstrated that during the course of infection, the CF environment selects for mutations in the QS regulatory gene, *lasR*. In addition, Singh et al. (2000) revealed that *P. aeruginosa* growing in laboratory biofilms and in the sputum of CF patients predominantly produce the *rhl* AHL, *N*-butyryl-L-homoserine lactone. This may indicate that the *rhl* signaling system plays a more prominent role in CF biofilms and, by losing the *las* system, the organism mutes some acute virulence functions, while maintaining expression of necessary *rhl*-encoded factors for establishing and maintaining chronic biofilms. Although the *rhl* system requires a functional *las* system for optimal expression (Van Delden and Iglewski 1998), secondary mutations can restore expression of the *rhl* system in a *lasR* mutant background.

Colonization of urinary catheters is another example of a *P. aeruginosa* chronic infection. Stickler et al. (1998) demonstrated that AHL signals were produced by *P. aeruginosa* biofilms growing on the surface of a catheter.

Involvement of two-component sensor kinases in the switch between acute and chronic *P. aeruginosa* infection

The genome of the opportunistic pathogen *P. aeruginosa* encodes over 60 two-component sensor kinases and uses several (including RetS, LadS and GacS) to reciprocally regulate the production of virulence factors involved in the development of acute or chronic infections (Stover et al. 2000). The hybrid sensors RetS and LadS have been shown to be involved in the switch between acute and chronic infections by antagonistically controlling expression of virulence genes, such as the type III secretion system-encoding genes, the genes that are required for biofilm formation and those involved in polysaccharide synthesis (Laskowski et al. 2004; Ventre et al. 2006; Goodman et al. 2009). These sensor kinases intersect with another two-component system formed by the GacS/GacA pair (Goodman et al. 2009). The GacS/GacA system regulates the expression of virulence factors, stress tolerance genes, enzymes for secondary metabolism, as well as periplasmic proteins important for motility. GacA is a positive transcriptional regulator of the *lasRI* and *rhlRI* operons, which are responsible for the enzymes that synthesize *N*-3-oxo-dodecanoyl-homoserine lactone and *N*-butanoyl-homoserine lactone, respectively (Venturi 2006).

It therefore appears that the multisensor signaling network consisting of RetS, LadS and GacS/GacA controls the expression of a significant number of *P. aeruginosa* virulence genes as well as the transition from acute to chronic infection.

Conclusion

Pseudomonas aeruginosa is a highly effective opportunistic pathogen that can cause a range of chronic and acute infections. The pathogenesis of *P. aeruginosa* infection is obviously multifactorial as suggested by the large number of both cell-associated and extracellular virulence factors and the broad spectrum of diseases due to the bacterium. After an initial colonization phase, mostly dependent on cell-associated virulence factors, the infectious process advances either to an acute infection or to a chronic infection characterized by different level of production of extracellular factors. Many of the extracellular virulence determinants required for tissue invasion and dissemination are controlled by QS systems that enable the bacteria to secrete these factors in a coordinated, cell-density-dependent manner and overwhelm the host defense mechanisms during acute infection. For some conditions, QS may be an integral part in building a biofilm community and thus contributes to pathogenesis of persistent pulmonary infections in CF patients. Additionally, biofilm formation may allow the high local cell densities necessary to achieve a quorum.

The QS represents an attractive therapeutic target because of the key role that it plays in the global regulation of multiple *P. aeruginosa* virulence factors and the importance of this role for the virulence of the organism in multiple different infections. In concert with currently used antipseudomonal antibiotics, inhibitors of QS may be potent drugs for the eradication of severe *P. aeruginosa* infections.

Finally, because of the contribution of many scientists around the world, the QS circuitry in *P. aeruginosa* belongs to one of the best understood bacterial cell-to-cell communication systems and now serves as model system for analysis of complex regulatory networks in microorganisms.

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