Chapter 41

Pseudomonas aeruginosa

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium. It has a pearlescent appearance and grape-like or tortilla-like odour. *P. aeruginosa* grows well at 25°C to 37°C, and its ability to grow at 42°C helps distinguish it from many other *Pseudomonas* species. *P. aeruginosa* is a ubiquitous microorganism which has the ability to survive under a variety of environmental conditions. It not only causes disease in plants and animals, but also in humans, causing serious infections in immunocompromised patients with cancer and patients suffering from severe burns and cystic fibrosis (CF).

Most strains of *P. aeruginosa* produce one or more pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown). Previous investigations have suggested that pyocyanin not only contributes to the persistence of *P. aeruginosa* in the lungs of CF patients, but also interferes with many mammalian cell functions, including cell respiration, ciliary beating, epidermal cell growth, calcium homeostasis and prostacyclin release from lung endothelial cells [1]. However, the precise molecular mechanism mediated by pyocyanin pathology is unknown.

P. aeruginosa strains produce two distinct types of O antigen (O-Ag): a common polysaccharide antigen (A-band) composed of a homopolymer of D-rhamnose, and an O-specific antigen (B-band) composed of a heteropolymer of three to five distinct sugars in its repeat units. So far, *P. aeruginosa* isolates have been classified into 20 serotypes by the International Antigenic Typing Scheme (IATS) [2]. The lipopolysaccharide (LPS) of *P. aeruginosa* is less toxic than that of other Gram-negative rods, facilitating its establishment of chronic infections by eliciting a low inflammatory response [3].

The genome of *P. aeruginosa* consists of a single circular chromosome. *P. aeruginosa* has a relatively large genome (5.5–7 Mb) and high G + C content (65–67%). Because of the large genome, *P. aeruginosa* encodes a large number of enzymes for various metabolic pathways, conferring high nutritional versatility. In addition, about 8% of the genome encodes regulatory genes, which enables the bacterium to adapt to complex growth environments.

PATHOGENESIS

P. aeruginosa encodes numerous virulence factors that enable it to establish various human infections. It has evolved a complicated regulatory network to control temporal and spatial expression of selected virulence factors for maximum benefit for bacterial survival.

Adhesins

Adhesion of *P. aeruginosa* to the host tissues is a crucial early step in infection and pathogenesis. Regardless of biotic or abiotic surfaces, *P. aeruginosa* exploit this property to colonize, replicate, overcome environmental shear forces and obtain nutrients. Generally, the surfaces that pathogens attach to are specific. *P. aeruginosa* uses cell-surface components or appendages to promote the attachment to cells or to inanimate surfaces. There are at least three different adherence factors or adhesins: type IV pili, mediating adhesion to epithelial host cells; flagella, binding to mucin on epithelial cells; and the core oligosaccharide of LPS, mediating adhesion to the cystic fibrosis transmembrane conductance regulator (CFTR) of epithelial cells.

Type IV Pili-Mediated Adhesion

P. aeruginosa surfaces have type IV pili (T4P) that are retractable and flexible filaments. Pili filaments are homopolymers of pilin protein and have an average diameter of 5.2 nm and length of 2.5 μm. T4P of *P. aeruginosa* have multiple functions, such as surface motility, biofilm and

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microcolony formation, adhesion, immune evasion, transformation of DNA, cell signalling and phage attachment. Additionally, T4P can trigger the host immune defences and is a vaccine target. Disruption of T4P results in reduced virulence of *P. aeruginosa* [4].

T4P play an important role in adherence to host epithelial cells. About 90% of the adhesion capability of *P. aeruginosa* to host cells is dependent on T4P. *P. aeruginosa* lacking T4P results in a loss of adherence to eukaryotic cells. There are two subfamilies of T4P, type IVa pili (T4aP) and type IVb pili (T4bP). T4bP include a subtype called the tight adherence pili (Tad). Each of the three classes of T4P has a distinct assembly system. T4aP are the dominant adhesins in adherence to the epithelial cells of the host. T4aP interact with the glycosphingolipids (GSLs), asialo GM1 and asialo GM2 on epithelial cell surfaces, and the receptor-binding site is buried in the D-region structure, which is only exposed at the tip of T4aP [5]. Recent studies have discovered T4P can also bind to N-glycans, as receptors on the apical surface of polarized epithelial host cells, to induce subsequent host PI3K/Akt signalling pathway and bacterial invasion [6].

Flagella-Mediated Mucin Binding

P. aeruginosa has a single polar flagellum which is composed of a flagellin named FliC, although there are a large number of genes involved in flagella assembly and function. Flagella in *P. aeruginosa* are required for multiple functions, such as adhesion, motility and biofilm formation. Flagella-defective *P. aeruginosa* displays reduced invasion of epithelial host cells and pathogenicity [7].

Several studies have shown that the receptors for flagella-mediated adhesion are mucins, which lubricate and protect tissues from pathogens. Mucins can be divided into secreted mucins and cell-associated mucins. Both types of mucin have been reported to bind flagella. Flagellin can also bind to asialo GM1 and Toll-like receptor 5 (TLR5) that activate the host innate immune system [8]. Recent studies have demonstrated that at the basolateral surface, flagella mediate adhesion to heparan sulphate (HS) chains of heparan sulphate proteoglycans (HSPGs) on polarized epithelial host cells, which results in the activation of the epidermal growth factor receptor (EGFR), and subsequently the PI3K/Akt signalling pathway and bacterial invasion [6].

Core Oligosaccharide (OS) of LPS-Mediated Binding of CFTR

Core OS is a branched oligosaccharide that contains nine or ten monosaccharides. Several studies have demonstrated that the core OS is the ligand for the CFTR receptor and mediates the internalization by epithelial cells. CFTR transports chloride ions across epithelial cell membranes and this channel plays an important role in maintaining the moderate mucus of epithelial cell membranes. A CFTR defect results in mucus overload, which favours the survival and propagation of *P. aeruginosa*. The *P. aeruginosa* LPS outer core specifically recognizes CFTR amino acids sequence 108−117, which is located in the first extracellular domain of CFTR. The interaction can activate the formation of membrane lipid rafts that contain CFTR and many other proteins for subsequent internalization of the *P. aeruginosa*. CFTR mutant has been shown to enhance infection and pathology when infected with *P. aeruginosa* in in vivo and in vitro conditions [9]. Conversely, the CFTR- and lipid raft-mediated internalization of bacterial cells in corneal epithelial cells has been demonstrated to develop keratitis caused by *P. aeruginosa* infection [10]. So the function of the core OS-specific binding to CFTR is both advantageous and disadvantageous, depending on the infection sites.

Secreted Toxins and Exoenzymes

P. aeruginosa has evolved a number of complex secretion systems, which deliver virulence factors either into the extracellular environment or into the host cell cytosol. Among the seven secretion systems in the Gram-negative bacteria that have been found so far, *P. aeruginosa* contains five secretion systems: type I, type II, type III, type V and type VI.

The type I secretion system (T1SS) is composed of an outer-membrane protein and an ABC (ATP-binding cassette) transporter. The proteins secreted by the T1SS have a C-terminal secretion signal. There are two types of T1SS in *P. aeruginosa*. The Apr system is involved in the extracellular secretion of the alkaline protease AprA, which is a virulence factor involved in various *P. aeruginosa* infections. The other T1SS involves utilization of iron and requires *has* genes. The secreted protein HasAp is a haemophore, which binds haem from haemoglobin. HasAp is considered to be a crucial component for *P. aeruginosa* survival in the early stages of infection [11].

The type II secretion system (T2SS) uses a two-step process to deliver extracellular proteins. The first step involves Sec- or Tat-dependent delivery from the cytosol into the periplasm and the second step involves T2SS complex-mediated further secretion into the extracellular space. There are two types of T2SS in *P. aeruginosa*: the Xcp (extracellular protein) and the Hxc (homologous to Xcp) systems. The proteins secreted by the T2SS include LasA, LasB, PrpL, exotoxin A and phospolipase C.

The type III secretion system (T3SS) can inject toxic proteins directly into the cytosol of eukaryotic cells. Upon cell−cell contact between bacterial and host cells, the virulence factors are injected directly into the cytosol of host

cells through needle-like bacterial surface structures, causing damage to the host cells.

The type V secretion system (T5SS) is, like the T2SS, a two-step process secretion system. Following Sec-dependent secretion into the periplasm, the proteins are secreted through the outer membrane utilizing the translocase function of the C-terminal portion of the protein (auto-transporters) or another helper protein (two-partner secretion). Proteins secreted by the T5SS include EstA, LepB and LepA.

The type VI secretion system (T6SS) is also a needle-like complex. Three T6SSs have been found in *P. aeruginosa*: HSI-I, HSI-II and HSI-III. The effectors for the HSI-I T6SS are Tse1, Tse2 and Tse3, which target other bacterial species, presumably for the purpose of competition in the environment [12]. Recent research has also found that the HSI-I T6SS needle is a dynamic contractile phage tail-like structure, protruding from the cytosol to the bacterial surface [13]. The HSI-II T6SS was shown to enhance bacterial internalization in epithelial cells [14], while the function of the HSI-III T6SS is not known.

Various toxins and exoenzymes are secreted through the above-mentioned secretion systems, including exotoxin A, rhaminolipid, elastases, alkaline protease and phospholipase C, all of which contribute to bacterial virulence.

Exotoxin A (PE) is one of the most important virulence factors in *P. aeruginosa*. It is a member of a class of enzymes termed mono-ADP-ribosyltransferases. The protein is secreted through the T2SS and enters into the eukaryotic cell by binding to a special receptor on the surface of the cell. ADP-ribosyltransferase activity of this protein modifies and inactivates elongation factor 2 (eEF-2), resulting in the inhibition of protein synthesis and cell death.

The structure of PE has been elucidated and has three structural domains. The N-terminal domain Ia (amino acids 1−252) is responsible for the receptor binding; domain II (amino acids 253−364) helps PE to cross cellular membranes; and domain III (amino acids 405−613) has ADP-ribosyltransferase activity [15] (Fig. 41.1). There are two important amino acid motifs in PE. One is located in domain II and exposed at the surface of the protein. The cleavage of the motif by eukaryotic protease furin is necessary for its toxic effect on eukaryotic cells. The second important motif is REDLK (amino acids 609−613) at the C-terminus of PE which is an endoplasmic reticulum retention sequence, also necessary for the toxicity of the protein.

Elastases are the most abundant among the many proteases that are secreted by *P. aeruginosa* and have a wide range of substrates. One of the substrates of elastases is elastin, which is the element of the connective tissue with high stability against most proteases.

Both LasA and LasB have elastolytic activity and are secreted by the T2SS of *P. aeruginosa.* The precursor of the extracellular LasA protein has a molecular weight of 40 kDa. The 40-kDa LasA can degrade elastin. A 22-kDa LasA fragment is also found in *P. aeruginosa* and was shown to enhance the elastolytic activity of *Pseudomonas* elastase and other proteases [16]. LasB protease is a neutral zinc metalloprotease. LasB is synthesized as a pre-proenzyme (53.6 kDa) with three domains: the signal peptide (2.6 kDa), the pro-peptide (18 kDa), and the mature, secreted protease (33 kDa). The protein uses an autoproteolytic mechanism to remove the pro-peptide, which is also necessary for its secretion [17].

Alkaline protease (AP) is a Zn^{2+}-metalloprotease, a member of the repeats in the toxin (RTX) family of proteins. AP can activate the epithelial sodium channel (ENaC) in CF [18]. It can also degrade human gamma interferon (IFN-γ), and inhibit opsonized zymosan-stimulated neutrophil oxygen consumption. Therefore, AP is an important virulence factor in *P. aeruginosa*. The secretion of AP depends on the T1SS in *P. aeruginosa*. AP consists of two domains, an N-terminal proteolytic domain (PD) with a metal-binding motif, and a C-terminal domain with a Ca^{2+}-binding motif. Recent research has found that Ca^{2+} binding can induce AP folding and secretion [19].

Phospholipase C (PLC) in eukaryotic cells catalyses the synthesis of diacylglycerol (DAG) from the hydrolysis of phosphatidylinositol or phosphatidylcholine (PC). The process of DAG metabolism is very important for various signalling processes, including apoptosis, oncogenesis and inflammation.

P. aeruginosa can secrete two different types of PLC, one is the haemolytic PLC (PlcH), and the other is the non-haemolytic PLC (PlcN). Recent research has

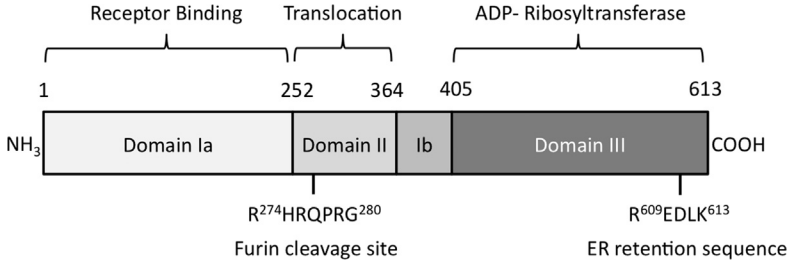

FIGURE 41.1 Schematic representation of the structural and functional domains of exotoxin A.

identified a new PLC, called PlcB. PlcH and PlcN are secreted by the T2SS and PlcB is secreted via the Sec pathway. Numerous studies indicate that PlcH is a virulence factor for mammals, plants, yeast and insects. It can degrade PC and sphingomyelin in eukaryotic cell membranes. PlcH is also responsible for the destruction of the lung surfactant [20].

Rhamnolipids are biosurfactants and are surface-active amphipathic molecules. Rhamnolipids are composed of mono- or dirhamnose linked to 3-hydroxy fatty acids of various chain lengths. They are glycosides and have three parts: a glycon part and an aglycon part linked to each other via an O-glycosidic linkage. The most frequently seen three rhamnolipids species are L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monorhamnolipid), L-rhamnosyl-L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (dirhamnolipid) and 3-(3-hydroxyalkanoyloxy)alkanoic acid (HAA) in *P. aeruginosa*. Rhamnolipid biosynthesis is carried out by gene products of *rlhA, rlhB* and *rhlC*, whose expression is regulated by a quorum sensing (QS) system.

The function of the rhamnolipids in *P. aeruginosa* is still not very clear. Rhamnolipids were initially identified as a heat-stable haemolysin which can affect the function of macrophages, and also mucociliary transport and ciliary beating [21]. Later, the rhamnolipids were found also to modulate swarming motility and affect biofilm architecture in *P. aeruginosa*. There is evidence that the rhamnolipids are required in the detachment from *P. aeruginosa* biofilms. Iron-limiting conditions promote twitching motility which is also due to increased rhamnolipid production [22].

Toxins Directly Injected into the Host Cells

P. aeruginosa is capable of causing various human tissue infections due in part to the numerous virulence factors mentioned above. Most importantly, *P. aeruginosa* also encodes a T3SS involved in the secretion of at least four effector proteins, including ExoS, ExoT, ExoU and ExoY, all of which contribute to cytotoxicity at different levels [23]. The T3SS is composed of more than 30 proteins that assemble into a 'needle-like' complex on the bacterial cell surface, designed to deliver effector molecules directly into the cytoplasmic compartments of host cells, ultimately evading host immune attack. Components of the type III secretion machinery share significant amino acid sequence homology with that of flagella and a structural similarity of the two systems has also been demonstrated [24]. The needle is presumed to be inserted into the host cell to inject the effector molecules.

The T3SS of *P. aeruginosa* has been shown to be an important virulence mechanism, affecting the bacterial infectivity in a burn model, an acute mouse pneumonia model and a rat lung infection model. In humans, infection with a type III-secreting isolate correlated with severe disease. There are four known effector molecules: ExoS and ExoT, both having an ADP-ribosyltransferase (ADPRT) activity and a GTPase-activating protein (GAP) activity; an acute cytotoxin ExoU with lipase activity; and an adenylate cyclase ExoY. The ExoS preferentially ADP-ribosylates several of the Ras family of GTP-binding proteins required for the regulation of intracellular vesicle transport, cell proliferation and differentiation. Its ADP ribosylating activity requires host 14-3-3 proteins which specifically interact with the C-terminus of the ExoS. The ADPRT activity of ExoS causes programmed cell death in various types of cultured cells [25,26]. In a mouse model of acute pneumonia, ExoS plays a significant role in bacterial persistence in the lung, dissemination, and causing mortality. Interestingly, the toxic effect of ExoS is dependent on its ADP-ribosylating activity.

P. aeruginosa strains harbouring the *exoS* gene are capable of inducing apoptosis at high frequency in epithelial and fibroblast cell lines. Mutants of the *exoS* gene failed to induce apoptosis, and further analysis showed that the ADP-ribosylating activity of ExoS is essential for inducing host cell apoptosis [26]. Apoptotic cells were clearly observed in human and animal tissues following infection by *P. aeruginosa* [27,28].

Apoptosis can either be advantageous or detrimental to the host throughout the course of infection. Generally, in infections involving intracellular pathogens, such as *Chlamydia* species, apoptosis of infected host cells favours clearance of bacterial pathogens. However, for extracellular pathogens, as in the case of *P. aeruginosa*, apoptosis of host inflammatory cells may be advantageous to the pathogens. A successful control of respiratory infection requires that the host maintain the right balance between apoptotic and anti-apoptotic pathways, as failure to do so may convert an acute self-limiting infection into a chronic disabling disease.

P. aeruginosa harbours multiple regulators to tightly control the expression of the large T3SS gene cluster, responding to environmental stimuli, such as low calcium and direct contact with host cells. Expression of the T3SS genes is under the control of a transcriptional activator, ExsA, belonging to the AraC family of transcriptional regulators. Normally, ExsA is bound by a repressor (ExsD) which inhibits its activator function. Under a type III- inducing condition, however, a small intracellular repressor protein (ExsE), normally bound to ExsC, is secreted from the cell through the type III secretion apparatus. Excess ExsC now binds ExsD, freeing ExsA to activate T3SS operons [29]. Without a functional type III secretion apparatus, expression of the whole T3SS regulon is repressed due to the accumulation of intracellular ExsE. How low calcium or host cell contact leads to the activation of type III secretion is not yet understood.

Mucoid Conversion (Alginate Overproduction)

P. aeruginosa is a leading cause of morbidity and mortality in CF patients. The most striking characteristic about strains infecting the CF pulmonary tract is the frequent conversion in vivo to highly mucoid colony morphology due to alginate overproduction. Alginate, a polymer of D-mannuronic and L-guluronic acid, allows *P. aeruginosa* to persist in the lungs of CF patients. It is considered to be the main cause of a poor prognosis and high mortality.

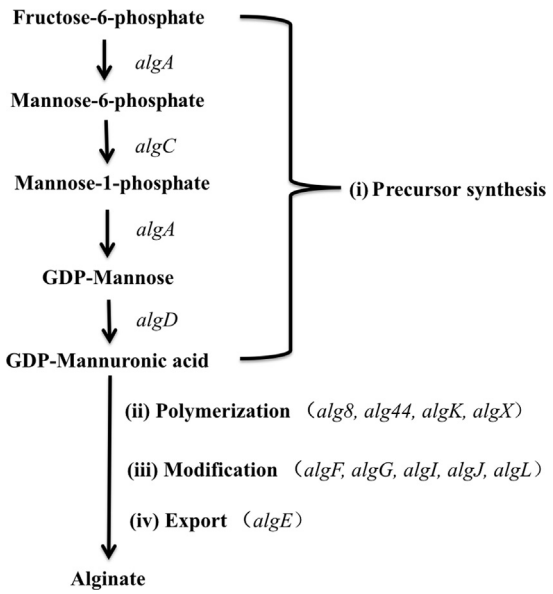

FIGURE 41.2 Alginate biosynthesis pathway and relevant genes.

Although alginate is not essential for all biofilms, it has notable effects on the biofilm architecture. Alginate protects *P. aeruginosa* from the host immune response by inhibiting complement activation and decreasing phagocytosis by neutrophils and macrophages, as well as by sequestering the free radicals that are released from these cells. Alginate seems to protect *P. aeruginosa* from various environmental stresses. Meanwhile, alginate overproduction influences the expression of other virulence factors, such as T3SS expression and flagellum. Recent research suggests a high level of coordinated regulation with respect to virulence, with AlgT/U serving a major central role [30].

Twelve genes, designated the *algD* operon, encode the core alginate biosynthesis machinery. These gene products are responsible for the four steps of alginate biosynthesis in *P. aeruginosa*: (i) precursor synthesis; (ii) polymerization and cytoplasmic membrane transfer; (iii) periplasmic transfer and modification; and (iv) export through the outer membrane (Fig. 41.2). Regulation of alginate synthesis is complex, involving many regulatory genes responding to various environmental cues.

AlgU/T or σ^{22} is essential for alginate production. The *algD* promoter is regulated by the σ^{22} factor and three transcription factors, AlgR, AlgB and AmrZ [31] (Fig. 41.3). MucA is an inner-membrane protein with one transmembrane domain and acts as an anti-sigma factor by binding to σ^{22}. The MucA encoding gene is in the same operon as *algU* where the *algU* gene is followed by four downstream genes called '*mucA*−*mucB*−*mucC*−*mucD*'. In response to environmental stress signals, MucA is degraded, resulting in the activation of σ^{22} and alginate overproduction. Several important proteases and mechanisms relate to the degradation of MucA (Fig. 41.3). MucA22 is a mutant

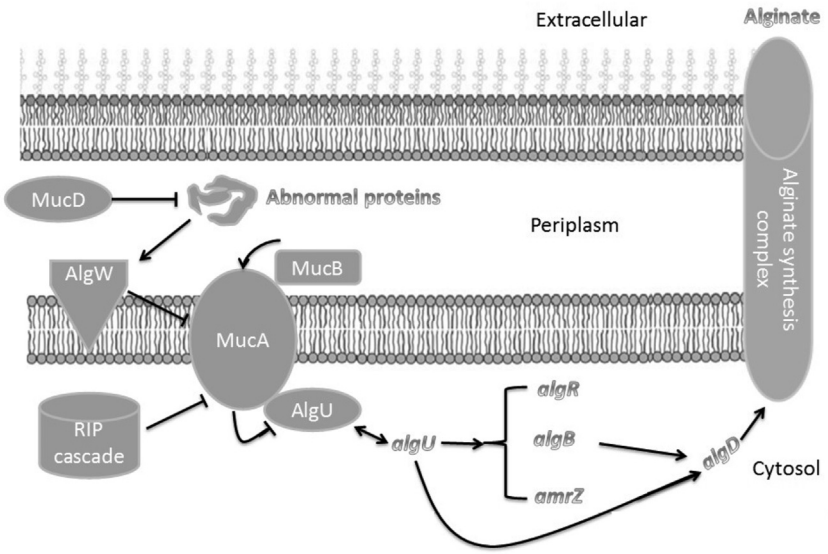

FIGURE 41.3 Regulation of alginate biosynthesis in *P. aeruginosa*.

variant commonly associated with mucoid isolates from CF patients. MucB is a negative regulator of σ^{22} factor, a periplasmic protein that binds the periplasmic domain of MucA, protecting it from proteolysis. The function of MucC has not been elucidated. The last gene in the operon is *mucD*, which encodes another negative regulator of alginate overproduction. MucD is a periplasmic protease involved in the proteolysis of abnormal proteins that is required for resistance to oxidative and heat stresses.

AlgR is a protein of the two-component regulator family, which can bind to three different sites upstream of the *algD* promoter. FimS is a sensory component that interacts with AlgR, encoded upstream of *algR*. *fimS-algR* seems to form one operon which is under the positive control of σ^{22} factor. FimS is not required for alginate production in mucoid *P. aeruginosa*, but FimS and AlgR are both required for type IV pilus. AlgR also controls hydrogen cyanide production, activates the expression of approximately 58 genes and represses the Rhl QS system. Therefore, AlgR is also a global regulator that not only regulates alginate synthesis but also other genes including virulence factors.

AlgB is needed for *algD* expression in mucoid *P. aeruginosa*. Downstream of *algB* is *kinB* which encodes the cognate environmental sensor for AlgB. The *algB*−*kinB* genes form an operon that is also under σ^{22} control. AlgB regulates alginate gene expression, and KinB is a negative regulator of alginate production in wild-type strains. Such regulation is triggered by degradation of MucA by activated intramembrane proteases AlgW and/or MucP [32]. KinB and AlgB also control a large number of genes, including those involved in carbohydrate metabolism, quorum sensing, iron regulation, rhamnolipid production and motility [32].

AmrZ (AlgZ) is also essential for alginate biosynthesis. AmrZ positively regulates type IV pilin expression and twitching motility, but negatively regulates flagellum biosynthesis and self-expression. Clearly, AmrZ functions as both a transcriptional activator and a repressor of multiple virulence factors of *P.* aeruginosa [33].

AlgW is a positive regulator of alginate overproduction. It is the functional homologue of *E. coli* DegS, which responds to misfolded or accumulated proteins in the periplasm, thus a model suggests membrane stress activates AlgW and alginate overproduction. AlgW has a PDZ domain, which is required for proteolysis of MucA, and an LA loop that prevents MucA binding. These two domains cooperate to regulate the proteolytic activity of AlgW [34].

Interaction with Other Virulence Factors

Mucoid conversion is always accompanied by lower toxicity. Many invasive virulence factors are down-regulated in mucoid isolates, such as by the T3SS, tissue-damaging proteases LasA and LasB, and flagellar motility. Recent research shows that it may be independent of alginate production, but dependent on AlgU. The cAMP/Vfr-dependent signalling (CVS) pathway is defective in *mucA* mutants, which depend on AlgU and AlgR, but do not depend on alginate overproduction. CVS regulates the expression of many invasive virulence factors, including T3SS, elastase, exotoxin A, T4P and protease IV.

Biofilm Formation

Biofilms show high tolerance to antibiotics and the host immune systems. For *P. aeruginosa*, biofilms formed in the CF lung result in antibiotic resistance and are believe to be the major cause of mortality. Biofilms formed by alginate-overproducing *P. aeruginosa* strains show highly structured architecture which is thought to result in persistence of *P. aeruginosa* in CF patients through promotion of resistance to antimicrobials and host immune systems.

Some *P. aeruginosa* strains cannot produce alginate but can form biofilm normally. The biofilms formed by these strains are composed of a polysaccharide matrix encoded by *psl* and *pel* loci. The *psl* locus encodes mannose-rich polysaccharide that plays an essential role in cell−surface and cell−cell interactions as well as biofilm formation [35]. *P. aeruginosa* can also form biofilm at a liquid−air surface, called pellicle. Pellicle is a glucose-rich polysaccharide and the *pel* locus contains genes for the synthesis of the glucose-rich component of the non-mucoid *P. aeruginosa* biofilm matrix.

DNA is also a major matrix component of *P. aeruginosa* biofilm. Researchers found that DNase treatment can inhibit biofilm formation in *P. aeruginosa* and even initiate the dispersal of mature biofilms. Further studies show that the DNA in biofilms is similar to *P. aeruginosa* genomic DNA, suggesting that this DNA may be the result of whole-cell lysis. This lysis is regulated by *P. aeruginosa* quinolone QS signals, as mutations in quinolone QS do not undergo autolysis, whereas high quinolone signals result in high autolysis.

QS plays an important role in *P. aeruginosa* biofilm formation. Among three known QS systems, the Las system is found to play an important role in the formation of mature biofilm in *P. aeruginosa*. The transcription of the *pel* operon decreases in the QS system defective strain and the QS system is also linked to swarming and DNA release. Biofilm formation and QS gene expression are maximal at low iron concentrations.

Antibiotic Resistance

Infections by *P. aeruginosa* are very difficult to eradicate due to their high resistance to antibiotics, which is

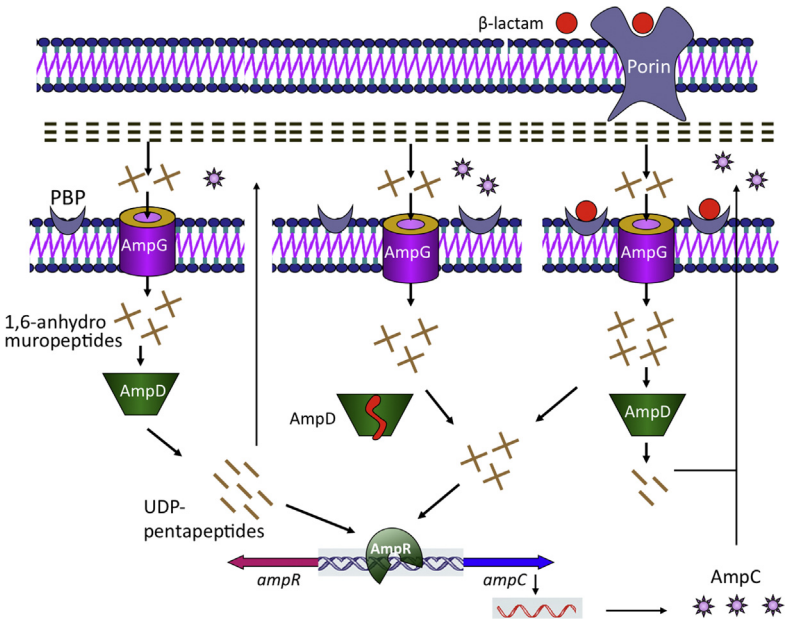

FIGURE 41.4 Activation of *ampC* gene expression under various growth conditions.

attributable to both intrinsic resistance and acquired resistance. The latter mainly refers to acquisition of resistance genes on mobile genetic elements, while the intrinsic resistance primarily refers to chromosomally encoded resistance mechanisms. The three most generally studied resistance mechanisms in *P. aeruginosa* are the resistance genes, efflux pumps and membrane impermeability.

Resistance Genes

ampC-Mediated Resistance to β-Lactams

All four classes of β-lactamases have been identified in *P. aeruginosa*. Two classes of β-lactamases are typically harboured on the chromosome: cephalosporinase, which is encoded by *ampC*, belongs to class C, while the other is a class D oxacillinase encoded by *poxB*. The expression of AmpC can be induced when encountering benzyl penicillin and narrow-spectrum cephalosporins. When the expressional level of AmpC is significantly increased, *P. aeruginosa* is resistant to almost all classes of β-lactams, except the carbapenems [36].

Two mechanisms contribute to the high expression level of *ampC*, induction and derepression. In the case of induction, when *P. aeruginosa* encounters specific β-lactams or β-lactamase inhibitors that can bind to penicillin-binding proteins (PBPs), the concentration of muropeptides is increased and its binding to AmpR converts AmpR into transcriptional activator for the *ampC* promoter, resulting in an increase in *ampC* expression (Fig. 41.4). In the case of derepression, mutations in the *ampD* structural gene are the cause, commonly observed in clinical isolates. In these strains, the AmpD amidase is modified or decreased, so the concentration of muropeptides in the cytoplasm permanently increases, culminating in a constitutive elevation of *ampC* expression (Fig. 41.4). Although not as common as *ampD*-related mutations, the mutated AmpR link to derepression of AmpC in *P. aeruginosa* has been described. The full derepression of AmpC in *P. aeruginosa* is complicated and not always a single-step process compared with the case in *Enterobacteriaceae*. AmpE, homologues of AmpD (AmpDh2 and AmpDh3), and PBP4 also are involved in the regulation of *ampC* expression [37,38]; these regulatory mechanisms are not well understood and need further study.

Genes Mediating Resistance to Aminoglycosides

Resistance to aminoglycoside is due to acquired or chromosomally encoded aminoglycoside-modifying enzymes (AMEs), which are common determinants in *P. aeruginosa* except in CF isolates where these mechanisms are uncommon [39]. Aminoglycoside modifications, leading to antibiotic inactivation, as well as rRNA methylases, play important roles in the resistance of aminoglycoside among clinical isolates. Some clinical strains of *P. aeruginosa* that are resistant to aminoglycoside are found to be carrying more than one modifying enzyme and exhibit broad-spectrum aminoglycoside resistance [40].

Efflux-Mediated Resistance

The reduction of drug accumulation in the cytoplasm is also an important mechanism to resist antibiotics. There

are two ways to achieve the purpose of reducing drug accumulation: one is through membrane impermeability, and the other is by membrane-associated pumps. Efflux pumps belonging to the resistance-nodulation-division (RND) family are the most significant contributors to antimicrobial resistance in *P. aeruginosa*, with a total of 12 RND systems encoded on the *P. aeruginosa* genome. RND pumps consist of a periplasmic membrane fusion protein (MFP), an outer-membrane factor (OMF), and a cytoplasmic membrane (RND) transporter.

The MexAB-OprM efflux pump was the first multidrug efflux pump discovered in *P. aeruginosa* and is most frequently linked to β-lactam resistance in clinical isolates. Besides β-lactams and β-lactamase inhibitors, MexAB-OprM is also able to export many other types of antibiotics, including tetracyclines, macrolides, fluoroquinolones, chloramphenicol, trimethoprim and novobiocin. MexAB-OprM is constitutively expressed in wild-type strains, and is involved in intrinsic resistance. Expression of *mexAB-oprM* is regulated directly or indirectly by three repressors, MexR, NalD and NalC. MexR binds as a stable homodimer to the *mexA* promoter and represses transcription of the *mexAB-oprM* operon. NalD also binds to the *mexA* promoter region but downstream of the *mexR* binding sites. NalC is an indirect repressor of MexAB-OprM [41]. Mutations in *mexR*, *nalC* and *nalD* have been reported in clinical isolates, but a mutational event within one of them may not be the sole mechanism to increase MexAB-OprM transcription.

The MexCD-OprJ efflux pump shares a high degree of similarity to MexAB-OprM. The substrates of MexCD-OprJ include tetracycline, fluoroquinolones, chloramphenicol, trimethoprim, novobiocin, macrolides and β-lactams. MexCD-OprJ does not have an extensive substrate profile for the β-lactams compared to MexAB-OprM, but it preferentially exports the fourth-generation cephalosporins. Transcription of *mexCD-oprJ* can be observed in wild-type cells, but the levels of protein cannot be detected. The expression of MexCD-OprJ is controlled by a single regulator, NfxB, which is located upstream of MexCD-OprJ but transcribes divergently from the operon. Binding of NfxB to the *nfxB-mexC* intergenic region negatively regulates the expression of MexCD-OprJ, as well as its own expression. Mutations in *nfxB* have been described in laboratory and clinical isolates, leading to the hyperexpression of MexCD-OprJ.

The MexEF-OprN efflux pump also shares amino acid homology with MexAB-OprM and MexCD-OprJ, but its regulation is unique. MexEF-OprN is not suppressed by a negative regulator. On the contrary, a transcriptional activator, MexT, belonging to the LysR family, is capable of positively regulating its expression. Unusually, wild-type strains carry inactivating mutations in the *mexT* gene. Additional *cis*-acting mutations or deletions within *mexT* can convert inactive MexT into an active form. In some strains, inactivating mutations are not present in *mexT*, stimulation of MexT requires mutations within a gene, *mexS*, which is located upstream of *mexT* and encodes a putative oxidoreductase/dehydrogenase homologue. It is believed that inactivation of MexS causes a build-up of metabolites that serve as effector molecules for MexT, which up-regulates *mexEF-oprN* expression to remove the toxic metabolites [42].

The MexXY efflux pump extrudes specific β-lactams (e.g. cefepime), aminoglycosides, fluoroquinolones, erythromycin, chloramphenicol and tetracycline. MexXY is the only pump of the 12 identified RND systems that mediates aminoglycoside resistance in *P. aeruginosa* PAO1. The *mexXY* system lacks a gene coding for an outer-membrane protein. It use OprM and possibly other outer-membrane proteins (OpmB, OpmG, OpmH and OpmI) to form a functional efflux pump. Deletion of *mexXY* increases susceptibility in wild-type strains, indicating that this pump is involved in intrinsic resistance. The expression of *mexXY* is controlled by a single regulator, *mexZ*, which locates upstream of *mexXY* but transcribes divergently from the operon. Binding of MexZ to the *mexZ-mexX* intergenic region negatively regulates the expression of *mexXY*. Mutations in *mexZ* or the *mexZ-mexX* intergenic region have been described in clinical isolates and associated with hyperexpression of *mexXY* [43].

Membrane Impermeability

All Gram-negative bacteria have an outer membrane that naturally prevents large, hydrophilic molecules from passing through. In order to get inside the cell, these molecules must pass through porins, which are protein channels that span the outer membrane and are water-filled. Sequence analysis of the *P. aeruginosa* genome has identified 163 known or predicted outer-membrane proteins, with 64 of these grouped into three families of porins [44]. Certain hydrophilic antibiotics, such as β-lactams, aminoglycosides, tetracyclines, some fluoroquinolones, and quinolones, can diffuse through porins. Conversely, dysfunction of these specific porin channels can lead to decreased susceptibility of *P. aeruginosa* to certain antibacterial agents. There are several different porin channels that contribute to the inherent resistance of *P. aeruginosa* to antimicrobial agents.

The major general porin in *P. aeruginosa* is OprF. The loss of OprF has not been found to be a major cause of antibiotic resistance. The loss of another porin, OprD, has been commonly linked with the resistance to carbapenems in clinical isolates [45]. The mechanisms involving OprD-mediated resistance include decreased transcription of *oprD* and mutations that disrupt the translational production of a functional porin for the outer membrane.

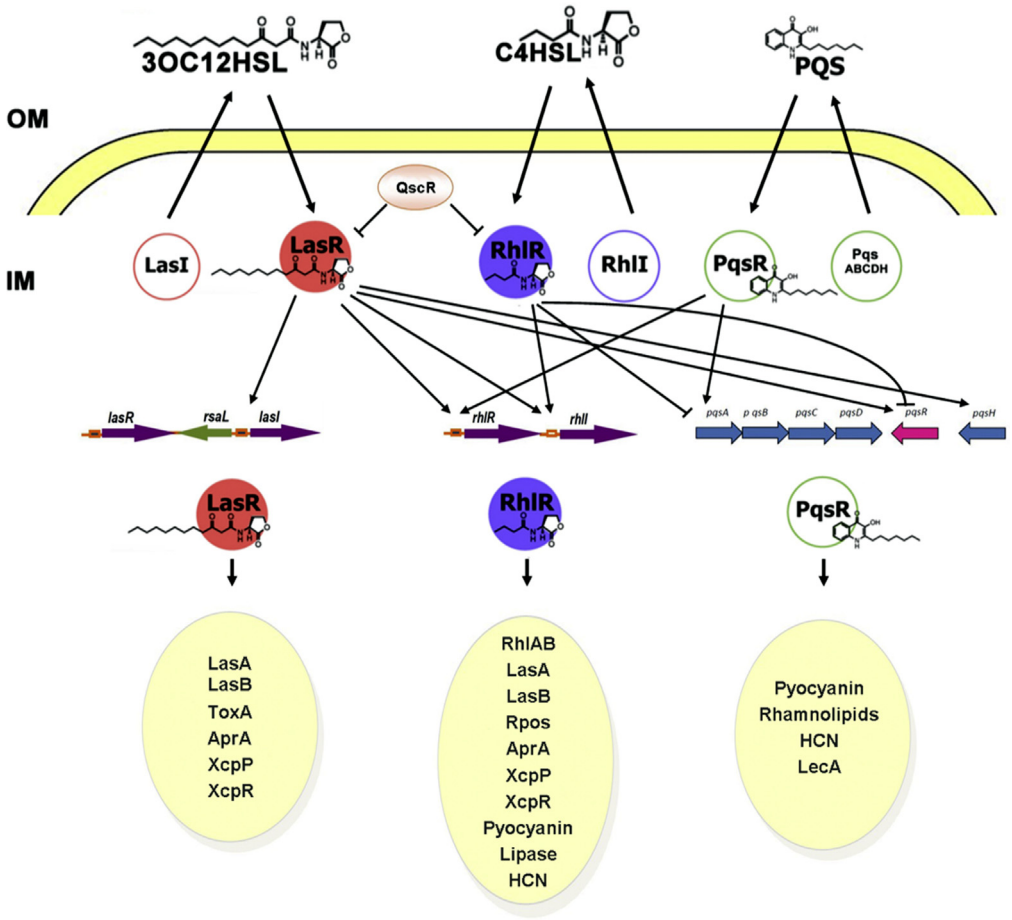

FIGURE 41.5 *P. aeruginosa* quorum sensing circuits. Three autoinducer (AI) synthases, LasI, RhlI and PqsABCDH, produce AI molecules of 3OC12HSL, C4HSL and PQS, respectively. The AIs are detected by the cytoplasmic transcription factors LasR, RhlR and PqsR, respectively, and activate the expression of indicated target genes.

In addition, it has been demonstrated that *P. aeruginosa* membrane permeability can be regulated. A novel two-component regulatory system (PprA and PprB) was identified and shown to regulate membrane permeability, but the downstream genes responsible for such phenotype have not been identified yet [46].

Regulation of Virulence Genes

The signalling network of *P. aeruginosa* is one of the most complex systems known to date, and is the best studied among all microorganism systems. *P. aeruginosa* relies on numerous signalling pathways to sense, respond, and adapt to fluctuating environmental cues, causing both acute and chronic infections in a variety of hosts. Acute infections are associated with motility and cytotoxicity via the T3SS, while chronic infections relate to biofilm formation and reduced virulence. During colonization of a susceptible host, *P. aeruginosa* has evolved a sophisticated and extensive array of regulatory networks to control the switch between planktonic and biofilm lifestyles and consequently influence whether they cause acute or chronic infections.

Quorum Sensing Regulation

P. aeruginosa harbours three QS systems: two LuxI/LuxR-type QS circuits (*las* and *rhl*) that are based on the intercellular signals *N*-acyl homoserine lactones (AHLs), and the non-LuxI/LuxR-type 2-heptyl-3-hydroxy-4-quinolone-based system called the *Pseudomonas* quinolone signalling (PQS) system (Fig. 41.5). Many traits controlled by these three QS systems are through the regulation of virulence factor production, biofilm maturation, and motility phenotypes. The *P. aeruginosa* QS systems are arranged hierarchically with the *las* system positively regulating both the *rhl* and *pqs* systems. Additionally, the *rhl* system negatively regulates the *pqs* system, while *pqs* autoinduces PQS synthesis and further activates *rhl* system expression. Moreover, each of these systems is further modulated by a

plethora of regulators that function at the transcriptional, translational and post-translational level.

AHL-Dependent QS Systems

The *las* and *rhl* systems use dedicated autoinducer synthases (LasI and RhlI) to produce autoinducers *N*-3-oxo-dodecanoyl homoserine lactone (3OC12HSL) and *N*-butyryl-HSL (C4HSL), respectively. When sufficient concentrations are achieved, the molecules recognize and activate their cognate receptors, LasR and RhlR, thereby altering the expression of multiple downstream virulence genes. The LasR−3OC12HSL complex activates transcription of target genes involved in acute infection and host cell damage, including those encoding the LasA and LasB elastases, exotoxin A, and alkaline protease. RhlR−C4HSL induces the expression of several genes, including those responsible for the production of rhamnolipids, and represses those responsible for assembly and function of the T3SS. Both the Las and Rhl systems contain an autoinducing feed-forward loop where their regulators (LasR and RhlR) induce transcription of their cognate synthase genes that allow rapid signal amplification. Further, a hierarchical relationship exists between the Las and Rhl systems: binding of the LasR−3OC12HSL complex directly up-regulates transcription of *rhlR* and *rhlI*. Thus, activation of the LasIR system promotes the later activation of the RhlIR system (Fig. 41.5).

Besides RhlR and LasR, genome sequencing has revealed *P. aeruginosa* possesses several LuxR homologues lacking a LuxI-type cognate partner, which have been designated as orphan LuxR homologues [47]. These highly conserved LuxR-type orphans may respond to endogenously synthesized AHL(s). An orphan LuxR homologue called QscR (quorum sensing control repressor) that can form mixed dimers with LasR and RhlR, rendering them inactive, has been reported. Thus, QscR likely controls a specific regulon that overlaps with the already overlapping LasR- and RhlR-dependent regulons and prevents aberrant QS responses before the cells reach 'a quorum'.

4-Quinolone-Dependent QS System

The third *P. aeruginosa* QS system utilizes 2-heptyl-3-hydroxy-4-quinolone as a signalling molecule, designated the *Pseudomonas* quinolone signal (PQS), to control virulence gene expression. PQS is produced by gene products encoded by the *pqsABCD* operon and *pqsH*. The PQS is then sensed and bound with high affinity by its cognate LysR-type receptor PqsR (also called MvfR) (Fig. 41.5). Interestingly, expression of *pqsH* and *pqsR* is positively controlled by LasR−3OC12HSL, whereas the *rhl* system negatively regulates *pqsABCD* and *pqsR*. PqsR−PQS autoinduces PQS synthesis and further activates *rhlI* and *rhlR* gene expression. Thus, the PQS circuit is intimately tied to the LasI/LasR and RhlI /RhlR QS systems and therefore influences virulence factor production.

PQS-dependent signalling is critical in the *P. aeruginosa* QS circuit. The *pqsABCD* gene products can direct synthesis of the precursor molecule HHQ, which is also capable of potentiating PqsR binding to the *pqsABCD* promoter. HHQ can be released into the extracellular medium and subsequently taken up by neighbouring cells, where it either is converted into PQS by PqsH or binds directly to PqsR, in both cases activating PqsR-dependent gene expression and virulence to levels similar to those observed in response to PQS itself. Furthermore, a *pqsH* mutant, which produces HHQ but completely lacks PQS, does not display a defect in PqsR-mediated gene expression or virulence, indicating that HHQ is also a functional QS molecule.

The Gac/Rsm Regulator Pathway

In addition to the AHL- and PQS-dependent QS systems, *P. aeruginosa* controls its environmental lifestyle and virulence phenotype via two-component regulatory systems, which are basically formed by two proteins: a histidine kinase (HK) and a response regulator (RR). *P. aeruginosa* encodes 55 HKs, 89 RRs and 14 HK-RR hybrids, forming over 60 two-component systems, and possesses one of the largest pools of two-component system proteins identified in any microorganism analysed thus far [48]. One of the critical two-component systems is GacS/GacA, which is central to the control of the course of *P. aeruginosa* infection by inversely regulating the expression of virulence factors associated with acute (T3SS, T4P) and chronic (exopolysaccharides, T6SS) disease and thus the switch between acute and chronic infections. The GacS/GacA system consists of a transmembrane hybrid sensor HK, GacS. Upon autophosphorylation, GacS transfers a phosphate group to its cognate response regulator (RR), GacA, which in turn promotes the expression of two small untranslated regulatory RNAs, RsmZ and RsmY. Binding of RsmZ and RsmY to the small RNA-binding protein RsmA activates the production of genes involved in biofilm formation, QS and T6SS, meanwhile repressing multiple genes involved in T3SS, type IV pili formation and iron homeostasis. In a mouse acute pneumonia model, loss of RsmA results in reduced colonization during the initial infection stages of acute infection but ultimately favours chronic infection and results in increased inflammation in the lungs of infected mice [49].

Several additional accessory regulators modulate the GacS/A system. Two sensor kinases, LadS and RetS, have been found to modulate gene expression via the GacS/GacA/RsmYZ pathway (Fig. 41.6). LadS (lost adherence sensor) phosphorylates GacS, while RetS

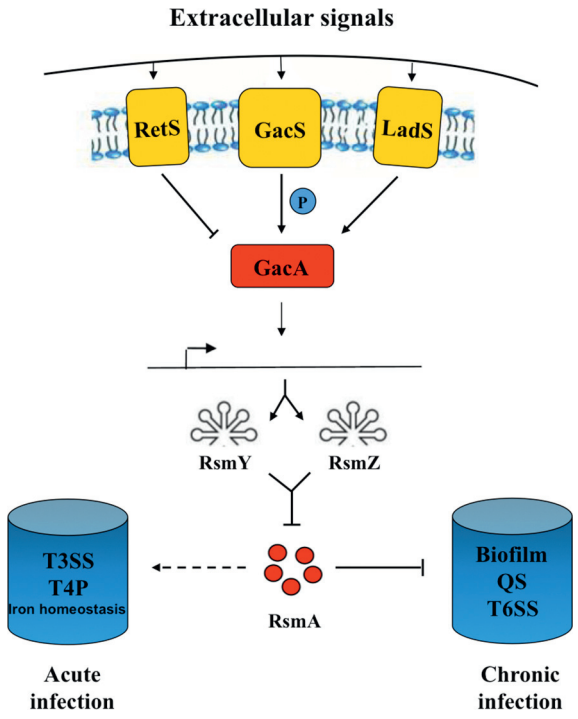

FIGURE 41.6 GAC regulatory network in *P. aeruginosa*. Small regulatory protein RsmA binds to the promoters of multiple genes, enhancing several acute virulence factors while repressing virulence factors associated with chronic infections. GacA phosphorylation via GacS stimulates the production of the small RNAs RsmZ and RsmY, which bind to the RsmA protein and inactivate it. 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 the LadS and GacS, forming a protein–protein complex with GacS that blocks RsmY and RsmZ production.

(regulator of exopolysaccharide and type III secretion) directly interacts with GacS and prevents GacS autophosphorylation, interfering with the subsequent phosphorylation of GacA and leading to a reduction in RsmYZ expression. In a mouse model of acute pneumonia, the *retS* mutant is unable to establish infection [50].

Nucleotide-Based Signalling Molecules

cAMP/Vfr Signalling

The *P. aeruginosa* PAO1 genome encodes three adenylate cyclases, the intracellular adenylate cyclases CyaA and CyaB, and a secreted effector, ExoY. Synthesis of cAMP is driven primarily by CyaB and to a lesser extent by CyaA. In *P. aeruginosa*, cAMP influences gene regulation through its activity as an allosteric activator of the CRP-homologous transcription factor Vfr (virulence factor regulator). Microarray analysis indicates that the cAMP–Vfr complex controls the expression of nearly 200 *P. aeruginosa* genes [51,52], and positively regulates the production of virulence factors important for acute infections, including: T4P, T3SS, T2SS, secreted toxins, degradative enzymes, and the *las* and *rhl* QS systems. In contrast, Vfr negatively regulates the expression of flagellar genes by repressing expression of *fleQ*, which encodes the master regulator of flagellar biogenesis. Modulation of cAMP homeostasis occurs via the Chp (chemotaxis-like chemosensory system) gene cluster in *P. aeruginosa*, where PilG, PilI, PilJ, ChpA, ChpC, FimL and FimV increase and PilH, PilK and ChpB reduce cAMP levels, establishing a link between Chp and T4P. Additionally, mutations in *mucA* and consequent activation of AlgU and the response regulator AlgR have been reported to inhibit cAMP–Vfr signalling, demonstrating that cAMP–Vfr signalling constitutes a complex signalling cascade with multiple regulatory inputs. Infection studies using a mouse pneumonia model with *vfr*, *cyaA* and *cyaB* mutants reveal an essential role for CyaB and Vfr during infection. ExoY is produced by *P. aeruginosa* and secreted via the T3SS directly into the cytoplasm of target host cells, where it modulates cAMP activity, thereby contributing to cytotoxicity.

ppGpp and pppGpp Signals

The second group of nucleotide-based signalling molecules 5′-diphosphate 3′-diphosphate guanosine (ppGpp) and 5′-triphosphate 3′-diphosphate guanosine (pppGpp) comprises the cellular alarmones. Under amino acid starvation conditions, these molecules rapidly accumulate in cells, triggering a switch from cell growth to survival adaptation. In *P. aeruginosa*, AlgQ up-regulates the production of the nucleoside diphosphate kinase Ndk, which is responsible for the production of these metabolites. Deletion of *algQ* leads to cell death in the late logarithmic phase, which is a clear consequence of the inability of this mutant to adapt from logarithmic growth to survival mode.

c-di-GMP Signalling

Another nucleotide-based signalling molecule, cyclic di-GMP (c-di-GMP), plays an important role as a secondary messenger molecule in many bacterial species. The intracellular concentration of c-di-GMP within a cell is fine-tuned by the opposing actions of two types of enzymes: diguanylate cyclases (DGC) containing GGDEF domains responsible for c-di-GMP synthesis; and phosphodiesterases (PDE) containing EAL or HD-GYP domains involved in c-di-GMP degradation. Several proteins have been identified that contain both GGDEF and EAL domains and thus may be capable of both synthesizing and degrading c-di-GMP, leading to the hypothesis that they act by balancing the internal cellular concentration of this molecule. Furthermore, a series of proteins in various bacterial species have been identified that contain degenerate GGDEF domains, suggesting that these

domains no longer generate c-di-GMP but instead function as allosteric sites or as c-di-GMP receptors. Together, these enzymes influence a wide range of phenotypes in diverse bacterial species including biosynthesis of adhesins and exopolysaccharides, motility, secretion, cytotoxicity, synthesis of secondary metabolites, and environmental stress adaptation. Modulation of c-di-GMP is associated with control of biofilm formation and other group behaviours.

ANTI-*PSEUDOMONAS* APPROACHES

The increasing antibiotic resistance of *P. aeruginosa* imposes a great threat to human health. At the same time, the development of new effective antibiotics has been slow, which is jeopardizing our fight against this bacterium. Thus, there is an urgent need for new treatment and prevention approaches. Presently, several new treatment strategies are in the pipeline, such as bacteriophages and agents targeting *P. aeruginosa* virulence factors. Research and clinical trials on vaccines against *P. aeruginosa* infection are underway.

Targeting Virulence

Traditional antibiotics inhibit the growth of or kill bacteria through inhibiting essential bacterial functions. Application of these antibiotics imposes a selection pressure on the bacteria, which leads to the rise of resistant strains. In addition, long-term use of antibiotics tends to destroy beneficial normal flora and induce superinfections, such as *Candida albicans* infections or *Clostridium difficile* colitis.

Inhibition of virulence factors disarms the pathogens, which leaves them vulnerable to the host immune system and eventually being cleared. Since the inhibition of virulence factors usually does not affect the growth of the bacteria, the chance for the development of antibiotic resistance is lower compared to traditional antibiotics. Due to the specificity, targeting virulence factors will not affect normal flora as much as traditional antibiotics.

Targeting Quorum Sensing

Numerous natural or synthetic compounds have been identified as QS inhibitors. Based on the structures of the autoinducers and corresponding receptors, compounds have also been synthesized to compete with the autoinducers for receptors. Most of these compounds have been shown to repress the production of QS-regulated virulence genes. Some compounds conferred protection in various infection models and interfered with biofilm formation. One QS inhibitor, garlic, has been evaluated in a small-scale clinical trial for the treatment of lung infections in cystic fibrosis patients and showed promising effects [53].

Targeting Biofilm

Efforts have been made to prevent the formation of biofilm and disrupt mature biofilm. For example, surfaces of medical instruments coated with *P. aeruginosa* attachment blocking compounds decreased the colonization of the bacteria and reduced the chances of ventilator-associated pneumonia [54,55]. Natural and synthetic compounds have been identified to prevent the formation of biofilm. Through degrading the matrix, and inducing bacterial dispersal, mature biofilm can be disrupted. QS inhibitors also reduce biofilm formation. When tested, some biofilm inhibitors, such as EDTA and alginate lyase, synergised with antibiotics in eradicating the biofilm [56,57].

Targeting Type III Secretion System

From large-scale screening of small-molecule libraries, Aiello et al. identified chemicals that inhibit the expression and secretion of T3SS effectors [58]. Chemicals that inhibit the ADP-ribosyltransferase activity of ExoS and the phospholipase activity of ExoU have also been identified [59,60]. These inhibitors protected cultured mammalian cells from T3SS-mediated cytotoxicity without affecting the growth of *P. aeruginosa*. In addition to natural and synthesized compounds, antibodies can also inhibit bacterial virulence factors. For example, antibody against PcrV blocks the translocation of effector proteins [61] and antibodies against flagella inhibit the swimming motility of the bacteria [62].

Vaccines and Immunotherapy

In the past decades, great efforts have been made to develop effective vaccines or therapeutic antibodies. However, an effective vaccine is not yet available on the market. Immunization against LPS or flagella resulted in varying degrees of failure. Tests of vaccines based on alginate, Psl, elastase, alkaline proteases, exotoxin A toxoid, OprF and OprI gave promising results that deserve further study.

The T3SS contains a needle tip complex, composed of PopD, PopB and PcrV, which is required for the translocation of T3SS effectors. Active and passive immunization with PcrV significantly improved the survival of *P. aeruginosa*-infected mice in a pneumonia or burn model [63]. Antibodies against PcrV blocked the translocation of T3SS effectors, which might be the primary mechanism for the protection [61]. PopB was recently identified to elicit Th17 responses. Intranasal immunization with PopB protected mice against *P. aeruginosa*

infection in an acute pneumonia model and IL-17 was shown to be essential for the protection [64]. Combination of PopB with other *P. aeruginosa* antigens might further improve the protective efficacy.

CONCLUDING REMARKS

P. aeruginosa is one of the most important opportunistic human pathogens, posing a major threat to individuals with underlying conditions, such as CF patients and immunocompromised individuals. Development of multidrug resistance is the major hurdle in the effective treatment of *P. aeruginosa* infections, as this bacterium possesses a high-level intrinsic antibiotic resistance as well as a tendency to develop acquired resistance. Various adaptive responses as well as genetic mutations contribute to the bacterial resistance against most commonly used antibiotics. Understanding the mechanisms of antibiotic resistance and their mode of evolution as well as transmission will help us to develop strategies to control drug resistance.

To develop countermeasures against *P. aeruginosa* infections, one important aspect of research is to better understand the mechanisms of pathogenesis, including the virulence mechanisms of acute and chronic infections, the signals and sensing mechanisms for switching from acute infection to chronic infection, especially its conversion into the biofilm mode of growth. The other aspect relates to the development of novel antimicrobial approaches, such as monoclonal antibodies targeting important virulence factors, effective vaccine development, and small molecules targeting the bacterial resistance mechanisms, such as increasing bacterial membrane permeability.

REFERENCES

[1] Caldwell CC, Chen Y, Goetzmann HS, Hao Y, Borchers MT, Hassett DJ, et al. *Pseudomonas aeruginosa* exotoxin pyocyanin causes cystic fibrosis airway pathogenesis. Am J Pathol 2009;175:2473–88.

[2] Bystrova OV, Knirel YA, Lindner B, Kocharova NA, Kondakova AN, Zahringer U, et al. Structures of the core oligosaccharide and O-units in the R- and SR-type lipopolysaccharides of reference strains of *Pseudomonas aeruginosa* O-serogroups. FEMS Immunol Med Microbiol 2006;46:85–99.

[3] Cigana C, Curcuru L, Leone MR, Ierano T, Lore NI, Bianconi I, et al. *Pseudomonas aeruginosa* exploits lipid A and muropeptides modification as a strategy to lower innate immunity during cystic fibrosis lung infection. PLoS One 2009;4:e8439.

[4] Hahn HP. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa*: a review. Gene 1997;192:99–108.

[5] Craig L, Pique ME, Tainer JA. Type IV pilus structure and bacterial pathogenicity. Nat Rev Microbiol 2004;2:363–78.

[6] Bucior I, Pielage JF, Engel JN. *Pseudomonas aeruginosa* pili and flagella mediate distinct binding and signaling events at the apical and basolateral surface of airway epithelium. PLoS Pathog 2012;8:e1002616.

[7] Dasgupta N, Wolfgang MC, Goodman AL, Arora SK, Jyot J, Lory S, et al. A four-tiered transcriptional regulatory circuit controls flagellar biogenesis in *Pseudomonas aeruginosa*. Mol Microbiol 2003;50:809–24.

[8] Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, et al. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature 2001;410:1099–103.

[9] Bajmoczi M, Gadjeva M, Alper SL, Pier GB, Golan DE. Cystic fibrosis transmembrane conductance regulator and caveolin-1 regulate epithelial cell internalization of *Pseudomonas aeruginosa*. Am J Physiol Cell Physiol 2009;297:C263–77.

[10] Zaidi T, Bajmoczi M, Zaidi T, Golan DE, Pier GB. Disruption of CFTR-dependent lipid rafts reduces bacterial levels and corneal disease in a murine model of *Pseudomonas aeruginosa* keratitis. Invest Ophthalmol Vis Sci 2008;49:1000–9.

[11] Delepelaire P. Type I secretion in gram-negative bacteria. Biochim Biophys Acta 2004;1694:149–61.

[12] Russell AB, Hood RD, Bui NK, LeRoux M, Vollmer W, Mougous JD. Type VI secretion delivers bacteriolytic effectors to target cells. Nature 2011;475:343–7.

[13] Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ. Type VI secretion requires a dynamic contractile phage tail-like structure. Nature 2012;483:182–6.

[14] Sana TG, Hachani A, Bucior I, Soscia C, Garvis S, Termine E, et al. The second type VI secretion system of *Pseudomonas aeruginosa* strain PAO1 is regulated by quorum sensing and Fur and modulates internalization in epithelial cells. J Biol Chem 2012;287:27095–105.

[15] Wedekind JE, Trame CB, Dorywalska M, Koehl P, Raschke TM, McKee M, et al. Refined crystallographic structure of *Pseudomonas aeruginosa* exotoxin A and its implications for the molecular mechanism of toxicity. J Mol Biol 2001;314:823–37.

[16] Peters JE, Galloway DR. Purification and characterization of an active fragment of the LasA protein from *Pseudomonas aeruginosa*: enhancement of elastase activity. J Bacteriol 1990;172:2236–40.

[17] McIver KS, Olson JC, Ohman DE. *Pseudomonas aeruginosa lasB1* mutants produce an elastase, substituted at active-site His-223, that is defective in activity, processing, and secretion. J Bacteriol 1993;175:4008–15.

[18] Butterworth MB, Zhang L, Heidrich EM, Myerburg MM, Thibodeau PH. Activation of the epithelial sodium channel (ENaC) by the alkaline protease from *Pseudomonas aeruginosa*. J Biol Chem 2012;287:32556–65.

[19] Zhang L, Conway JF, Thibodeau PH. Calcium-induced folding and stabilization of the *Pseudomonas aeruginosa* alkaline protease. J Biol Chem 2012;287:4311–22.

[20] Wargo MJ, Ho TC, Gross MJ, Whittaker LA, Hogan DA. GbdR regulates *Pseudomonas aeruginosa plcH* and *pchP* transcription in response to choline catabolites. Infect immun 2009;77:1103–11.

[21] Johnson MK, Boese-Marrazzo D. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginosa*. Infect immun 1980;29:1028–33.

[22] Glick R, Gilmour C, Tremblay J, Satanower S, Avidan O, Déziel E, et al. Increase in rhamnolipid synthesis under iron-limiting conditions influences surface motility and biofilm formation in *Pseudomonas aeruginosa*. J Bacteriol 2010;192:2973–80.

[23] Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. Nat Rev Microbiol 2009;7:654–65.
[24] Hueck CJ. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol Mol Biol Rev 1998;62:379–433.
[25] Jia J, Alaoui-El-Azher M, Chow M, Chambers TC, Baker H, Jin S. c-Jun NH_2-terminal kinase-mediated signaling is essential for *Pseudomonas aeruginosa* ExoS-induced apoptosis. Infect Immun 2003;71:3361–70.
[26] Kaufman MR, Jia JH, Zeng L, Ha UW, Chow M, Jin SG. *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of ExoS. Microbiology 2000;146:2531–41.
[27] Hotchkiss RS, Dunne WM, Swanson PE, Davis CG, Tinsley KW, Chang KC, et al. Role of apoptosis in *Pseudomonas aeruginosa* pneumonia. Science 2001;294:1783.
[28] Rajan S, Cacalano G, Bryan R, Ratner AJ, Sontich CU, van Heerckeren A, et al. *Pseudomonas aeruginosa* induction of apoptosis in respiratory epithelial cells: analysis of the effects of cystic fibrosis transmembrane conductance regulator dysfunction and bacterial virulence factors. Am J Respir Cell Mol Biol 2000;23:304–12.
[29] Yahr TL, Wolfgang MC. Transcriptional regulation of the *Pseudomonas aeruginosa* type III secretion system. Mol Microbiol 2006;62:631–40.
[30] Balasubramanian D, Kong KF, Jayawardena SR, Leal SM, Sautter RT, Mathee K. Co-regulation of β-lactam resistance, alginate production and quorum sensing in *Pseudomonas aeruginosa*. J Med Microbiol 2011;60:147–56.
[31] Wozniak DJ, Ohman DE. Transcriptional analysis of the *Pseudomonas aeruginosa* genes *algR*, *algB*, and *algD* reveals a hierarchy of alginate gene expression which is modulated by *algT*. J Bacteriol 1994;176:6007–14.
[32] Damron FH, Owings JP, Okkotsu Y, Varga JJ, Schurr JR, Goldberg JB, et al. Analysis of the *Pseudomonas aeruginosa* regulon controlled by the sensor kinase KinB and sigma factor RpoN. J Bacteriol 2012;194:1317–30.
[33] Pryor Jr EE, Waligora EA, Xu B, Dellos-Nolan S, Wozniak DJ, Hollis T. The transcription factor AmrZ utilizes multiple DNA binding modes to recognize activator and repressor sequences of *Pseudomonas aeruginosa* virulence genes. PLoS Pathog 2012;8:e1002648.
[34] Cezairliyan BO, Sauer RT. Control of *Pseudomonas aeruginosa* AlgW protease cleavage of MucA by peptide signals and MucB. Mol Microbiol 2009;72:368–79.
[35] Ma L, Jackson KD, Landry RM, Parsek MR, Wozniak DJ. Analysis of *Pseudomonas aeruginosa* conditional Psl variants reveals roles for the Psl polysaccharide in adhesion and maintaining biofilm structure postattachment. J Bacteriol 2006;188:8213–21.
[36] Sanders CC, Sanders Jr WE. Type I β-lactamases of gram-negative bacteria: interactions with β-lactam antibiotics. J Infect Dis 1986;154:792–800.
[37] Moya B, Dotsch A, Juan C, Blazquez J, Zamorano L, Haussler S, et al. β-lactam resistance response triggered by inactivation of a nonessential penicillin-binding protein. PLoS Pathog 2009;5:e1000353.
[38] Juan C, Moya B, Perez JL, Oliver A. Stepwise upregulation of the *Pseudomonas aeruginosa* chromosomal cephalosporinase conferring high-level β-lactam resistance involves three AmpD homologues. Antimicrob Agents Chemother 2006;50:1780–7.
[39] Islam S, Oh H, Jalal S, Karpati F, Ciofu O, Hoiby N, et al. Chromosomal mechanisms of aminoglycoside resistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Clin Microbiol Infect 2009;15:60–6.
[40] Poole K. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2005;49:479–87.
[41] Cao L, Srikumar R, Poole K. MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the *nalC* gene encoding a repressor of PA3720-PA3719. Mol Microbiol 2004;53:1423–36.
[42] Sobel ML, Neshat S, Poole K. Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. J Bacteriol 2005;187:1246–53.
[43] Vogne C, Aires JR, Bailly C, Hocquet D, Plesiat P. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrob Agents Chemother 2004;48:1676–80.
[44] Hancock RE, Brinkman FS. Function of *Pseudomonas* porins in uptake and efflux. Annu Rev Microbiol 2002;56:17–38.
[45] Li H, Luo YF, Williams BJ, Blackwell TS, Xie CM. Structure and function of OprD protein in *Pseudomonas aeruginosa*: from antibiotic resistance to novel therapies. Int J Med Microbiol 2012;302:63–8.
[46] Wang Y, Ha U, Zeng L, Jin S. Regulation of membrane permeability by a two-component regulatory system in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2003;47:95–101.
[47] Fuqua C. The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. J Bacteriol 2006;188:3169–71.
[48] Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 2000;406:959–64.
[49] Mulcahy H, O'Callaghan J, O'Grady EP, Macia MD, Borrell N, Gomez C, et al. *Pseudomonas aeruginosa* RsmA plays an important role during murine infection by influencing colonization, virulence, persistence, and pulmonary inflammation. Infect Immun 2008;76:632–8.
[50] Goodman AL, Kulasekara B, Rietsch A, Boyd D, Smith RS, Lory S. A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa*. Dev Cell 2004;7:745–54.
[51] Wolfgang MC, Lee VT, Gilmore ME, Lory S. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. Dev Cell 2003;4:253–63.
[52] Suh SJ, Runyen-Janecky LJ, Maleniak TC, Hager P, MacGregor CH, Zielinski-Mozny NA, et al. Effect of *vfr* mutation on global gene expression and catabolite repression control of *Pseudomonas aeruginosa*. Microbiology 2002;148:1561–9.
[53] Smyth AR, Cifelli PM, Ortori CA, Righetti K, Lewis S, Erskine P, et al. Garlic as an inhibitor of *Pseudomonas aeruginosa* quorum sensing in cystic fibrosis–a pilot randomized controlled trial. Pediatr Pulmonol 2010;45:356–62.

[54] Zodrow KR, Schiffman JD, Elimelech M. Biodegradable polymer (PLGA) coatings featuring cinnamaldehyde and carvacrol mitigate biofilm formation. Langmuir 2012;28:13993–9.

[55] Kollef MH, Afessa B, Anzueto A, Veremakis C, Kerr KM, Margolis BD, et al. Silver-coated endotracheal tubes and incidence of ventilator-associated pneumonia: the NASCENT randomized trial. JAMA 2008;300:805–13.

[56] Banin E, Brady KM, Greenberg EP. Chelator-induced dispersal and killing of *Pseudomonas aeruginosa* cells in a biofilm. Appl Environ Microbiol 2006;72:2064–9.

[57] Lamppa JW, Griswold KE. Alginate lyase exhibits catalysis-independent biofilm dispersion and antibiotic synergy. Antimicrob Agents Chemother 2013;57:137–45.

[58] Aiello D, Williams JD, Majgier-Baranowska H, Patel I, Peet NP, Huang J, et al. Discovery and characterization of inhibitors of *Pseudomonas aeruginosa* type III secretion. Antimicrob Agents Chemother 2010;54:1988–99.

[59] Arnoldo A, Curak J, Kittanakom S, Chevelev I, Lee VT, Sahebol-Amri M, et al. Identification of small molecule inhibitors of *Pseudomonas aeruginosa* exoenzyme S using a yeast phenotypic screen. PLoS Genet 2008;4:e1000005.

[60] Lee VT, Pukatzki S, Sato H, Kikawada E, Kazimirova AA, Huang J, et al. Pseudolipasin A is a specific inhibitor for phospholipase A_2 activity of *Pseudomonas aeruginosa* cytotoxin ExoU. Infect Immun 2007;75:1089–98.

[61] Sawa T, Yahr TL, Ohara M, Kurahashi K, Gropper MA, Wiener-Kronish JP, et al. Active and passive immunization with the *Pseudomonas* V antigen protects against type III intoxication and lung injury. Nat Med 1999;5:392–8.

[62] Campodonico VL, Llosa NJ, Grout M, Doring G, Maira-Litran T, Pier GB. Evaluation of flagella and flagellin of *Pseudomonas aeruginosa* as vaccines. Infect Immun 2010;78:746–55.

[63] Moriyama K, Wiener-Kronish JP, Sawa T. Protective effects of affinity-purified antibody and truncated vaccines against *Pseudomonas aeruginosa* V-antigen in neutropenic mice. Microbiol Immunol 2009;53:587–94.

[64] Wu W, Huang J, Duan B, Traficante DC, Hong H, Risech M, et al. Th17-stimulating protein vaccines confer protection against *Pseudomonas aeruginosa* pneumonia. Am J Respir Crit Care Med 2012;186:420–7.