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Review

Quorum sensing in bacterial virulence

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Bacteria communicate through the production of diffusible signal molecules termed autoinducers. The molecules are produced at basal levels and accumulate during growth. Once a critical concentration has been reached, autoinducers can activate or repress a number of target genes. Because the control of gene expression by autoinducers is cell-density-dependent, this phenomenon has been called quorum sensing. Quorum sensing controls virulence gene expression in numerous micro-organisms. In some cases, this phenomenon has proven relevant for bacterial virulence *in vivo*. In this article, we provide a few examples to illustrate how quorum sensing can act to control bacterial virulence in a multitude of ways. Several classes of autoinducers have been described to date and we present examples of how each of the major types of autoinducer can be involved in bacterial virulence. As quorum sensing controls virulence, it has been considered an attractive target for the development of new therapeutic strategies. We discuss some of the new strategies to combat bacterial virulence based on the inhibition of bacterial quorum sensing systems.

Introduction

Bacteria can communicate by producing and responding to small diffusible molecules that act as signals. These molecules have been termed autoinducers (AIs). AIs are produced at basal levels and their concentration increases with growth. Because the signals can diffuse through membranes, their concentration inside cells approximates the concentration in the environment. Upon reaching a critical concentration, the signal molecules can bind to and activate receptors inside bacterial cells. These receptors can then alter gene expression to activate behaviours that are beneficial under the particular condition encountered. As this phenomenon occurs in a cell-density-dependent manner, it has been termed quorum sensing (Antunes & Ferreira, 2009; Bassler & Losick, 2006; Fuqua *et al.*, 1994; 2001; Fuqua & Greenberg, 2002).

Many classes of AIs have been described to date. The most intensely studied AIs are the *N*-acylhomoserine lactones (AHLs) of Gram-negative bacteria, the peptides of Gram-positive bacteria and a class of AIs termed AI-2, whose structures remain unknown in most cases (Antunes & Ferreira, 2009). AHLs are usually detected through binding to and activation of cytoplasmic receptor proteins, which dimerize upon signal detection and can bind to promoter regions of target genes to activate or repress their transcription (Fuqua *et al.*, 1994). Peptides are usually detected through binding to membrane sensor proteins of the two-component system family, although some can also be transported to the cytoplasm before interacting with their receptors (Novick & Geisinger, 2008; Pottathil & Lazazzera, 2003). On the other hand, AI-2 binds a periplasmic protein and then interacts with either a two-component system or a transporter depending on the organism (Ng & Bassler, 2009; Taga *et al.*, 2001). Binding to a membrane-associated sensor kinase causes the activation of a phosphorelay cascade, which results in the activation or repression of a response regulator, culminating in altered gene expression (Ng & Bassler, 2009; Novick & Geisinger, 2008).

Quorum sensing was originally described in the marine luminescent bacterium *Vibrio fischeri*, where it functions as the control mechanism of light production and numerous other traits (Antunes *et al.*, 2007; Eberhard *et al.*, 1981; Engebrecht *et al.*, 1983; Engebrecht & Silverman, 1984). For years, it was thought that this phenomenon was limited to a few marine organisms but it is now widely recognized that many bacterial species utilize quorum sensing as part of their regulatory machinery (Antunes & Ferreira, 2009;

Abbreviations: 3OC12-HSL, *N*-3-oxododecanoyl-homoserine lactone; AE, attaching effacing; AHL, *N*-acylhomoserine lactone; AI, autoinducer; AIP, autoinducing protein; AQ, 2-alkyl-4-quinolone; C4-HSL, *N*-butyryl-homoserine lactone; CF, cystic fibrosis; EHEC, enterohaemmorhagic *E. coli*; HHQ, 2-heptyl-4-quinolone; IL, interleukin; LEE, locus of enterocyte effacement; PIA, polysaccharide intercellular adhesin; PQS, *Psuedomonas* quinolone signal; RIP, RNAIII-inhibiting peptide; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SRH, *S*-ribosyl-homocysteine; TNF-α, tumour necrosis factor alpha.

Bassler & Losick, 2006; Lyon & Novick, 2004; Smith *et al.*, 2006). Of interest, we now know that bacterial virulence is in many cases controlled by quorum sensing (Antunes & Ferreira, 2009). This has led to a burst in quorum sensing research and the role of quorum sensing in the virulence of multiple human pathogens has been studied in molecular detail. Here, we review the role of quorum sensing in bacterial pathogenicity using several model organisms. We discuss the quorum sensing mechanisms used to control virulence gene expression, the evidence that they are important during host infection and the available strategies to control bacterial virulence using quorum sensing inhibitors.

Quorum sensing in *Staphylococcus aureus* virulence

Many Gram-positive bacteria utilize peptide quorum sensing systems to control gene expression and *S. aureus* has served as a model to study bacterial peptide signalling (Novick & Geisinger, 2008). This organism is a member of the human microbiota and is found in approximately 30 % of the adult population (George & Muir, 2007; Roux *et al.*, 2009). Despite its widespread prevalence in healthy subjects, *S. aureus* is also a very dangerous opportunistic pathogen which has been increasingly associated with antibiotic resistance (George & Muir, 2007). *S. aureus* has multiple virulence factors and can display very rapid transmission, aiding its importance as a human pathogen (Massey *et al.*, 2006). Additionally, *S. aureus* forms biofilms on many surfaces, including indwelling devices such as urethral stents (Kehinde *et al.*, 2004). These indwelling devices, and subsequent biofilms formed on them, pose a serious risk for *Staphylococcus* infection (Balaban *et al.*, 2007).

One of the factors which contribute to *S. aureus* virulence is its peptide-based quorum sensing system, encoded by the accessory gene regulator (*agr*) locus (Novick & Geisinger, 2008) (Fig. 1). The autoinducer in the *agr* system is an oligopeptide that has been termed the autoinducing peptide (AIP), encoded by *agrD*. AIP is trimmed and secreted by AgrB, a membrane-bound protein (Ji *et al.*, 1995; Saenz *et al.*, 2000; Zhang *et al.*, 2004). The active AIP is 7–9 aa, with a 5-membered thiolactone ring (Roux *et al.*, 2009). AgrC is a membrane-bound sensor kinase to which extracellular AIP binds, leading to AgrC autophosphorylation and activation of AgrA (Ji *et al.*, 1995; Koenig *et al.*, 2004; Lina *et al.*, 1998). The *agr* system is intricately involved in the regulation of virulence genes, predominantly from two promoters, P2 and P3, which produce RNAII and RNAIII, respectively (Morfeldt *et al.*, 1995; Novick & Geisinger, 2008). P2 promotes the transcription of the *agr* operon from the RNAII transcript, which includes *agrA*, *agrB*, *agrC* and *agrD* (Novick *et al.*, 1995; Roux *et al.*, 2009). Active AgrA may be a phosphorylated homodimer that induces transcription at the P2 and P3 promoters, with a higher affinity for P2 (George & Muir, 2007; Koenig *et al.*, 2004). Transcription from P3 leads to the production of RNAIII, which is the effector molecule of the *agr* system (Roux *et al.*, 2009). RNAIII is a 514 nt regulatory RNA, which also functions as the mRNA for the δ-toxin (Balaban & Novick, 1995; Benito *et al.*, 2000; Kong *et al.*, 2006; Novick *et al.*, 1995). The 5′ end is thought to upregulate α-haemolysin, while the 3′ end is required for the repression of protein A synthesis (Kong *et al.*, 2006; Morfeldt *et al.*, 1995). RNAIII reduces the expression of surface adhesins, and increases the production of capsule, toxins and proteases (Novick & Geisinger, 2008; Roux *et al.*, 2009).

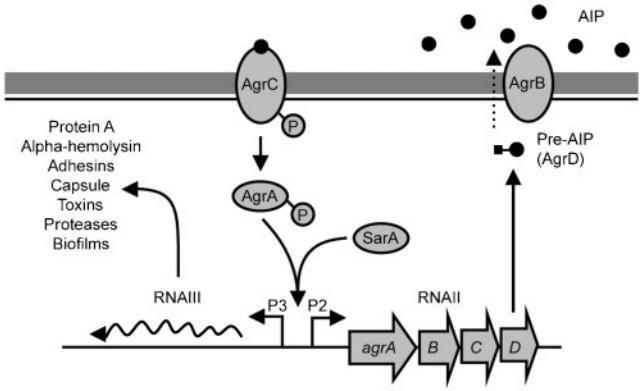

Fig. 1. The accessory gene regulator (*agr*) quorum sensing system of *S. aureus*.

The *agr* system is thought to regulate over 70 genes, 23 of which are known virulence factors (George & Muir, 2007). Of the virulence factors regulated by *agr*, there are two classes: the first class contains virulence factors involved in attachment to the host and immune evasion, while the second class contains genes involved in the production of exoproteins associated with invasion and toxin production (Bowden *et al.*, 2005; Yao *et al.*, 2006). It has been thought that the activation of the *agr* system essentially switches the bacterium from an adhesive, colonizing commensal to an invasive and aggressive pathogen (Roux *et al.*, 2009).

Four distinct groups of *agr* polymorphism have been identified, these are categorized as I–IV (George & Muir, 2007; Ji *et al.*, 1997). Each group has a distinct AIP, which is able to bind to the receptor from all groups. However, each AIP is only able to activate the receptor from the same group (Ji *et al.*, 1997). Apart from groups I and IV, which are able to cross-activate, all other groups are cross-inhibiting, i.e. an AIP from group II can inhibit the receptor from a group III strain (George & Muir, 2007; Mayville *et al.*, 1999; Otto *et al.*, 1998). There does appear to be a correlation between the *agr* group and the relative fitness of the *S. aureus* strain in an insect model of infection (Fleming *et al.*, 2006). Therefore, there is potential for therapies which are based upon cross-group inhibition (George & Muir, 2007). There remains no doubt that the

agr system is involved in *S. aureus* virulence; however, exactly how it contributes remains controversial.

One of the ways in which *agr* is thought to impact virulence is through its role in biofilm formation. The formation of biofilms by bacteria is a multi-step developmental process that starts with adhesion to a surface. Attached bacteria divide and give rise to macrocolonies. These macrocolonies later develop into mature biofilms, which can assume multiple topographies. The last step in biofilm development is detachment, which may be important for dissemination during an infectious process (Parsek & Tolker-Nielsen, 2008). It is thought that *S. aureus* possesses two independent mechanisms of biofilm formation; the first involves an extracellular polysaccharide, polysaccharide intercellular adhesin (PIA), and the second is thought to be PIA-independent, possibly involving adhesive proteins and the *sarA* and *agr* global regulators (Lauderdale *et al.*, 2009; Novick & Geisinger, 2008). Because biofilms are thought to play a critical role in *S. aureus* infection, the role of *agr* in biofilm formation has been explored. When *agr* is non-functional, *S. aureus* has enhanced adhesion abilities (Vuong *et al.*, 2000). Therefore, when *agr* is not active, the bacteria remain in the first stage of biofilm formation, adhering to a surface. However, *agr* is also important for detachment of clusters of cells from the biofilm (Kong *et al.*, 2006). An *agr* mutant strain has a detachment defect, and the detachment of bacterial cells from biofilms was found to coincide with *agr* expression (Kong *et al.*, 2006; Yarwood *et al.*, 2004). In addition, an *agr* mutant has increased adherence and more robust formation of static biofilms than its *agr*-containing counterparts (Novick & Geisinger, 2008). It is thought that this role of *agr* is brought about by the reduction in adhesin production and an increase in the production of both δ-haemolysin and proteases (Novick & Geisinger, 2008). Thus, an important role of quorum sensing in *S. aureus* is the regulation of biofilm formation, a central factor in *S. aureus* virulence.

The exact contribution of *agr* to disease remains somewhat controversial. Many studies have found that *agr* is required for virulence in models of septic arthritis, osteomyelitis, endophthalmitis and pulmonary infections (Abdelnour *et al.*, 1993; Booth *et al.*, 1997; Gillaspy *et al.*, 1995; Heyer *et al.*, 2002). However, other evidence seems to question the role of the *agr* system in virulence in other models (Cheung *et al.*, 1994; Kielian *et al.*, 2001). In cystic fibrosis (CF) patients with *S. aureus* infections, the *agr* system was inactive, suggesting that *agr* activation may not be necessary for infection (Goerke *et al.*, 2000). Through a temporal analysis of *agr* expression, Wright *et al.* (2005) have found that *agr* expression varies during different steps of infection. This suggests that *agr* may play different roles during the course of infection and this may explain the discrepancy in some of the results regarding its role in bacterial virulence. Interestingly, specific *agr* groups have been associated with some diseases, such as toxic shock syndrome, while other diseases, such as infections of CF patients, are associated with all four *agr* groups (George & Muir, 2007).

RNAIII is the effector of the *agr* system and is involved in bacterial virulence, so mechanisms to inhibit RNAIII have received considerable attention. An RNAIII-inhibiting peptide (RIP) has been found to inhibit *S. aureus* biofilm formation and toxin production (Cirioni *et al.*, 2007). It is thought that by inhibiting cell–cell communication, RIP is able to prevent adherence and virulence of *S. aureus*. Preliminary studies have shown that RIP has no adverse effects on laboratory animals (Balaban *et al.*, 2007; Cirioni *et al.*, 2007). Treatment with RIP and teicoplanin prevented *S. aureus* growth in a urethral stent model (Cirioni *et al.*, 2007). To further support the efficacy of RIP, Balaban *et al.* (2007) found that when RIP was administered at the time of infection, a concentration-dependent reduction in bacterial load was observed. It was also found that treatment with RIP 2 days post-infection suppressed bacterial growth and was more efficacious with multiple doses.

Quorum sensing control of *Pseudomonas aeruginosa* virulence

P. aeruginosa is a Gram-negative bacterium capable of surviving in a wide range of environments. This organism is an opportunistic pathogen and it is commonly associated with nosocomial infections and infections of severely burned individuals, and is a leading cause of death in severe respiratory infections, such as chronic lung infections in CF patients (Bendiak & Ratjen, 2009; Bodey *et al.*, 1983; Koch & Hoiby, 1993). Infections with *P. aeruginosa* are difficult to eradicate, due to their high levels of antibiotic resistance and growth in biofilms (Driscoll *et al.*, 2007).

At least three intertwined quorum sensing systems and one orphan autoinducer receptor affect the ability of *P. aeruginosa* to cause disease (Fig. 2). Two of these systems, *las* and *rhl*, rely on the production of AHLs as the signalling molecules (AIs) (De Kievit & Iglewski, 2000). In the *las* system, *N*-3-oxododecanoyl-homoserine lactone (3OC12-HSL) is produced by the enzyme encoded by the *lasI* gene. When *P. aeruginosa* reaches a certain threshold density, 3OC12-HSL binds to the transcriptional activator LasR. LasR, in turn, dimerizes and binds to target promoters to control gene expression (De Kievit & Iglewski, 2000). Similarly, in the *rhl* system, the *rhlI* gene encodes the enzyme involved in the production of *N*-butyryl-homoserine lactone (C4-HSL). As with 3OC12-HSL, C4-HSL binds to its cognate transcriptional regulator, RhlR, to control the activity of target promoters (De Kievit & Iglewski, 2000). The *rhl* system is controlled by the *las* system at both transcriptional and post-transcriptional levels (Latifi *et al.*, 1996). Besides LasR and RhlR, *P. aeruginosa* encodes an orphan receptor protein, QscR, which can sense 3OC12-HSL to control its own regulon (Chugani *et al.*, 2001; Fuqua, 2006; Schuster & Greenberg, 2006).

Multiple *P. aeruginosa* virulence factors are involved in the development of disease, including secreted factors (such as

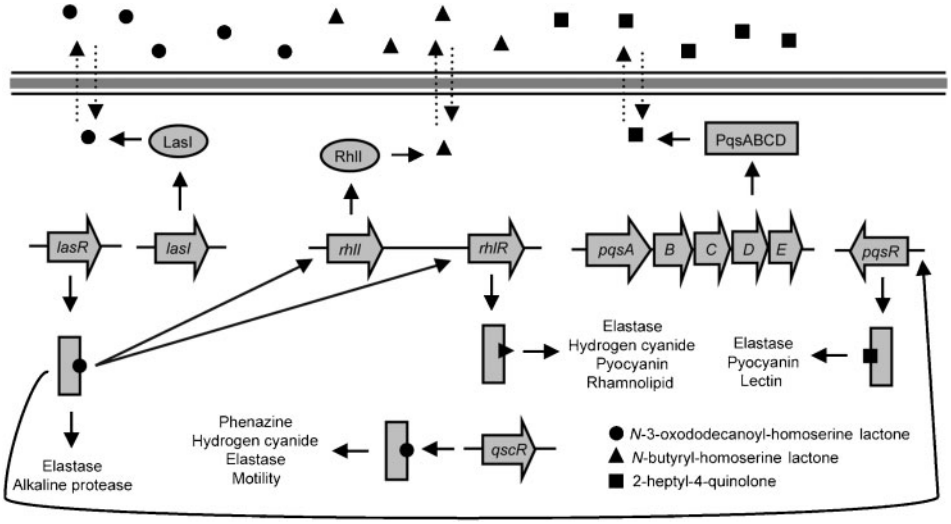

Fig. 2. Quorum sensing control of gene expression in *P. aeruginosa*.

proteases) and cell-associated factors (such as lipopolysaccharide and flagella), as well as the ability to form biofilms (Lyczak *et al.*, 2000). Quorum sensing regulates the production of several extracellular virulence factors, promotes biofilm maturation and regulates the expression of antibiotic efflux pumps, meaning that it has a key role in the pathogenesis of *P. aeruginosa* (Dekimpe & Déziel, 2009; Diggle *et al.*, 2006; Fuqua *et al.*, 2001; Schuster & Greenberg, 2006; Swift *et al.*, 2001; Wagner *et al.*, 2003; Whitehead *et al.*, 2001). The *las* and *rhl* systems regulate the timing and production of multiple virulence factors, including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins and superoxidase dismutase (Schuster *et al.*, 2003; Smith & Iglewski, 2003). The expression of these two quorum sensing systems has also been linked to the regulation of biofilm formation. Quorum sensing signalling may start in the early stages of biofilm development, which is characterized by microcolony formation, where *lasI* mutants are unable to form structurally normal biofilms (Davies *et al.*, 1998). Expression of the *lasI* gene is maximal at day 4 of biofilm development, decreasing between days 6 and 8. The expression of *rhlI* fluctuates during biofilm development and phenotypes of biofilm development with a *rhlI* mutant vary, according to the media and model used, supposedly due to different iron levels present (Davies *et al.*, 1998; De Kievit *et al.*, 2001; Patriquin *et al.*, 2008; Yoon *et al.*, 2002).

Significant concentrations of the AI molecules 3OC12-HSL and C4-HSL have been detected in sputum of CF patients colonized with *P. aeruginosa* (Erickson *et al.*, 2002; Singh *et al.*, 2000). This may indicate that quorum sensing is active during *P. aeruginosa* colonization of CF patients; however, it is important to note that quorum-sensing-deficient *P. aeruginosa* strains are often isolated from CF patients (Erickson *et al.*, 2002; Karatuna & Yagci, 2010; Le Berre *et al.*, 2008; Schaber *et al.*, 2004). This has spurred a major discussion in the scientific community about whether quorum sensing is really important during CF infections. It has been hypothesized that the maintenance of a functional quorum sensing system is a metabolic burden for *P. aeruginosa* and that co-colonization with quorum-sensing-proficient and -deficient strains is in the best interest of this community of pathogens (Heurlier *et al.*, 2006; Kohler *et al.*, 2009). Moreover, social exploitation in *P. aeruginosa* communities may provide an explanation for the emergence of quorum-sensing-deficient strains in human infections (Sandoz *et al.*, 2007). Nevertheless, it has been shown that *P. aeruginosa rhlI* and *lasI* mutants cause less tissue destruction and decrease mortality when compared with wild-type strains in multiple animal models (Smith & Iglewski, 2003), indicating an important role for quorum sensing in *P. aeruginosa* pathogenesis.

Apart from regulating the expression of virulence factors, some of the AIs have been shown to directly interact with host cells. 3OC12-HSL induces interleukin (IL)-8 secretion from human bronchial epithelial cells (DiMango *et al.*, 1995) and induces COX-2 production (Smith *et al.*, 2002). It also inhibits lymphocyte proliferation, down-regulates the production of tumour necrosis factor alpha (TNF-α) and IL-12, and activates T cells to produce gamma-interferon (Skindersoe *et al.*, 2009; Smith *et al.*, 2002). 3OC12-HSL also induces apoptosis in macrophages and neutrophils, suggesting that this molecule not only enhances the expression of virulence genes but also affects immune responses to infection (De Kievit & Iglewski, 2000; Smith *et al.*, 2002; Smith & Iglewski, 2003; Tateda *et al.*, 2003; Telford *et al.*, 1998). Additionally, 3OC12-HSL has been shown to directly affect one of the major regulators of immune responses, NFκB (Kravchenko *et al.*, 2008).

A third *P. aeruginosa* AI molecule, 2-heptyl-3-hydroxyl-4-quinolone (*Pseudomonas* quinolone signal; PQS) has also been identified (Déziel *et al.*, 2004; Lepine *et al.*, 2004; Pesci *et al.*, 1999). PQS belongs to the 2-alkyl-4-quinolone (AQ) family and it is synthesized via the *pqsABCD* genes in the *pqsABCDE* operon, which are responsible for the synthesis of 2-heptyl-4-quinolone (HHQ), the immediate PQS precursor (Diggle *et al.*, 2006). Both molecules have been shown to play a role in cell–cell communication in *P. aeruginosa*. PqsH, which is controlled by the *las* system (Gallagher *et al.*, 2002), plays a role in converting HHQ to PQS (Déziel *et al.*, 2004). PqsE is in an operon with *pqsA–D*, but is not involved in the synthesis of PQS. However, disruption of *pqsE* causes loss of signal transduction of several but not all of the processes believed to be regulated by the *pqs* system (Diggle *et al.*, 2007b). The exact mechanism of action of this protein is still unknown. However, it has been shown recently that PqsE can activate the transcription of PQS-controlled genes in the absence of PqsR and PQS by enhancing the *rhl* system (Farrow *et al.*, 2008). PqsR is a LysR transcriptional factor that is activated by HHQ and PQS, leading to the positive activation of many virulence factors, which include a large number of genes also controlled by *las* and *rhl*. In addition, PqsR also controls the *pqs* operon itself, generating a positive feedback loop (Cao *et al.*, 2001; Déziel *et al.*, 2004, 2005; Xiao *et al.*, 2006). Interestingly, the expression of PqsR is positively regulated by the *las* system (Xiao *et al.*, 2006). RlhR, in turn, affects the expression of the *pqs* system (Hazan *et al.*, 2010; McGrath *et al.*, 2004; Xiao *et al.*, 2006).

Through transcriptome analysis, over 90 genes were found to be regulated by the *pqs* system (Bredenbruch *et al.*, 2006; Déziel *et al.*, 2005). PQS has been shown to affect biofilm formation and to regulate several virulence factors in *P. aeruginosa*, including elastase, pyocyanin and LecA lectin, and it is considered essential for full virulence in multiple hosts (Cao *et al.*, 2001; Rahme *et al.*, 1997, 2000). PQS has been found in sputum, bronchoalveolar fluid and mucopurulent fluid from CF patients, suggesting that it may play an important role during the infection process (Collier *et al.*, 2002). Besides controlling bacterial virulence, PQS and HHQ have been shown to downregulate the host immune response through NFκB (Kim *et al.*, 2009b). Mutants that do not secrete these molecules have been shown to elicit increased TNF-α and IL-6 expression in cultured cells (Kim *et al.*, 2009b). In a mouse infection model, a *pqsA* mutant was unable to disseminate in the lung tissue as well as the wild-type strain, suggesting a role of these molecules in *P. aeruginosa* pathogenesis (Kim *et al.*, 2009b). PQS can also act as an iron chelator, and both the synthesis of PQS and the activity of PqsR–PQS are involved in iron homeostasis, another indication of the global importance of quinolone signalling for *P. aeruginosa* (Bredenbruch *et al.*, 2006; Oglesby *et al.*, 2008).

Because of the significant role of quorum sensing in the regulation of hundreds of virulence factors in *P. aeruginosa*, significant efforts have been made to discover molecules that inhibit quorum sensing in this organism. Natural quorum sensing inhibitors include cyclic sulfur compounds (Persson *et al.*, 2005), halogenated furanones (Givskov *et al.*, 1996), patulin and penicillin acid (Rasmussen *et al.*, 2005b), among others. Through the use of a screening system, garlic extracts and 4-nitropyridine-*N*-oxide were also identified as quorum sensing inhibitors (Rasmussen *et al.*, 2005a). These molecules specifically inhibited the activation of virulence genes by quorum sensing, reduced biofilm tolerance to tobramycin and also decreased virulence in a *Caenorhabditis elegans* pathogenesis model. In a recent study, a screening of quorum-sensing-inhibiting molecules derived from the plant species *Combretum albiflorum* revealed that catechin has a negative impact on the production of quorum-sensing-dependent virulence factors (Vandeputte *et al.*, 2010). Subinhibitory concentrations of macrolides have been shown to be effective in inhibiting quorum-sensing-dependent virulence both *in vivo* and *in vitro* (Imamura *et al.*, 2004; Molinari *et al.*, 1993; Sofer *et al.*, 1999; Tateda *et al.*, 2001). Furthermore, CF patients showed significant improvement in pulmonary function with the use of macrolides without any effect on the total *Pseudomonas* population (Fujii *et al.*, 1995; Saiman *et al.*, 2003). Despite its success in decreasing *P. aeruginosa* virulence, macrolides are not ideal because, like any other antibiotic, excessive usage is associated with bacterial resistance. Nevertheless, based on the results obtained with natural quorum sensing inhibitors, a number of new inhibitory molecules have been synthesized and used with some success (Kim *et al.*, 2008, 2009a; Liu *et al.*, 2010), demonstrating that quorum sensing inhibition is an attractive therapeutic target.

Quorum sensing in *Escherichia coli* virulence

Another bacterial species that uses quorum sensing to control virulence gene expression is *E. coli*. This organism produces a signal molecule termed AI-2 (Surette & Bassler, 1998). AI-2 was originally identified as one of the AIs controlling light production by the marine bacterium *Vibrio harveyi* (Bassler *et al.*, 1993). By utilizing a *V. harveyi* reporter strain that does not produce AI-2 but can respond to it, Surette and colleagues have identified *E. coli* strains that could activate the *V. harveyi* AI-2 sensor (Surette & Bassler, 1998). Subsequently, the gene responsible for AI-2 production was identified and named *luxS* (Surette *et al.*, 1999). Although the structure of AI-2 in *E. coli* is currently unknown, the molecule produced by the close relative *Salmonella enterica* serovar Typhimurium has been identified as (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (Miller *et al.*, 2004).

Genetic studies in enterohaemorrhagic *E. coli* (EHEC) and enteropathogenic *E. coli* revealed that LuxS controls the expression of the type-3 secretion system encoded by the locus of enterocyte effacement (LEE) pathogenicity island (Sperandio *et al.*, 1999). This important virulence determinant is required for the formation of the characteristic

attaching and effacing (AE) lesions caused by these pathogens (Celli *et al.*, 2000). Additionally, transcriptomic studies have revealed that LuxS is a global regulator in EHEC, controlling the expression of over 400 genes (DeLisa *et al.*, 2001; Sperandio *et al.*, 2001). Many of these genes have functions related to bacterial virulence such as flagellar motility, surface adhesion and Shiga toxin production.

Originally, it was believed that the phenotypes of *luxS* mutants could be attributed to the lack of AI-2 production. However, Sperandio *et al.* (2003) showed that the molecule responsible for the regulation of LEE and flagellar genes in *E. coli* is not AI-2 (Sperandio *et al.*, 2003). Because *luxS* controls the expression of LEE, it was expected that a *luxS* mutant would be unable to produce AE lesions in cultured epithelial cells. However, the mutant was fully proficient in inducing the formation of such lesions (Sperandio *et al.*, 2003). Through a series of elegant experiments, the authors showed that although the signal responsible for the regulation of LEE was dependent on *luxS*, it was not AI-2, but a new molecule. This new AI was then named AI-3 (Sperandio *et al.*, 2003) (Fig. 3).

The regulatory cascade linking AI-3 sensing and virulence gene expression in *E. coli* is extremely complex. Microarray studies of a *luxS* mutant have revealed that multiple regulatory genes are controlled by quorum sensing, and some of these genes have been characterized in further detail (DeLisa *et al.*, 2001; Sperandio *et al.*, 2001). QseBC is a bacterial two-component signalling system that is regulated by LuxS, with QseB being the response regulator and QseC the sensor kinase (Sperandio *et al.*, 2002b). Similar to LuxS, it has been found that QseBC controls motility gene expression (Sperandio *et al.*, 2002b). Mutant analyses have led to the conclusion that AI-3 affects gene expression through QseBC, since this AI activates *qseBC* expression and a *qseC* mutant is unable to regulate motility in response to AI-3 (Sperandio *et al.*, 2003). Biochemical studies have revealed not only that the expression of QseC is activated by AI-3 but also that this sensor kinase is the receptor for the AI (Clarke *et al.*, 2006). Interestingly, QseC also functions as the receptor for the host catecholamine hormones epinephrine and norepinephrine, indicating that small molecule signalling pathways in eukaryotes and bacteria can intertwine (Clarke *et al.*, 2006). Addition of AI-3 or catecholamines to QseC-containing liposomes resulted in an increased phosphorylation of this protein. Additionally, the activation of QseC resulted in phosphorylation of QseB, showing that QseB acts as the cognate response regulator in this system (Clarke *et al.*, 2006). Besides QseBC, multiple regulators are involved in the control of virulence gene expression. A second two-component regulatory system that controls the formation of AE lesions has been recently described (Reading *et al.*, 2009). This system is composed of the histidine kinase QseE and the response regulator QseF. These two proteins are involved in the transcriptional control of the effector EspFu, which is translocated into host cells by EHEC. Interestingly, a third gene in the same operon, *qseG*, separates *qseE* and *qseF*. QseG is an outer-membrane protein and it is also required for the translocation of type III secretion system effectors into host cells (Reading *et al.*, 2009). Besides these two-component systems, two LysR-type regulators, QseA and QseD, are also required for the control of LEE expression, revealing that a complex regulatory cascade links quorum sensing and virulence gene expression in *E. coli* (Sperandio *et al.*, 2002a; Walters & Sperandio, 2006).

The observation that quorum sensing controls the expression of multiple genes involved in *E. coli* pathogenicity has raised the hypothesis that this signalling system must be important for virulence *in vivo*. To test this hypothesis, Clarke *et al.* (2006) used a rabbit model of infection to show that a *qseC* mutant is attenuated during infection of rabbits, confirming the role of quorum sensing in *E. coli* virulence. Additionally, *qseC* has proved important for bacterial virulence in both *Salmonella enterica* serovar Typhimurium and *Francisella tularensis* (Rasko *et al.*, 2008).

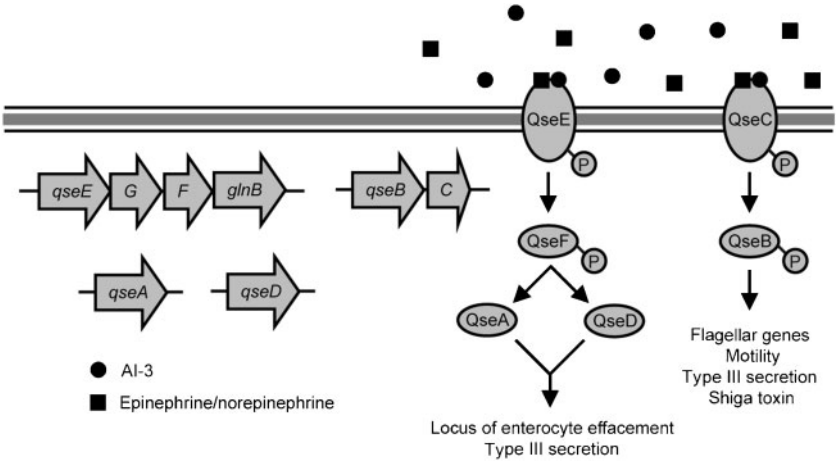

Fig. 3. Control of virulence gene expression through quorum sensing and adrenergic signalling in *E. coli*.

AI-3 control of virulence in *E. coli* has prompted investigators to develop small molecules that could inhibit this signalling system. To this end, Rasko *et al.* (2008) have recently described the identification of one such molecule. Through a high-throughput screening of a large library of compounds, the authors identified *N*-phenyl-4-{[(phenylamino)thioxomethyl]amino}-benzenesulfonamide (LED209) as an inhibitor of QseC and bacterial virulence, both *in vitro* and *in vivo*. LED209 inhibits QseC autophosphorylation, virulence factor production and AE lesion formation by EHEC. Additionally, LED209 can inhibit virulence factor production and host colonization by *Salmonella* Typhimurium and *F. tularensis* (Rasko *et al.*, 2008).

Additional roles of bacterial AIs

Although bacterial AIs have been studied for decades due to their signalling roles, these molecules can often perform other functions. Perhaps the clearest example of this comes from the fact that AI-2 is not only a signal molecule but also a metabolic by-product (Rezzonico & Duffy, 2008; Schauder *et al.*, 2001). AI-2 is synthesized through reactions involving the enzymes Pfs and LuxS. *S*-Adenosylmethionine (SAM) acts as a methyl donor in bacterial cells, creating *S*-adenosylhomocysteine (SAH). Because SAH is toxic, bacteria have evolved mechanisms to recycle it. This is accomplished through the action of the enzyme Pfs, which converts SAH to *S*-ribosylhomocysteine (SRH). SRH is then converted to AI-2 and homocysteine by LuxS (Schauder *et al.*, 2001). These findings indicate that the connection of AI-2 synthesis with bacterial metabolism is twofold. First, the precursor of AI-2 is a major methyl donor in bacterial cells, acting in multiple metabolic processes. Also, LuxS is involved in the detoxification of a metabolic intermediate. Therefore, the role of AI-2 as a bona fide signal has been questioned (Winzer *et al.*, 2002). Although such a role is clear in organisms such as *E. coli* and *V. harveyi*, it is likely that in most cases, the production of AI-2 is a mere consequence of SAM utilization and SAH detoxification. This could explain why LuxS is widespread in bacteria whereas AI-2 receptors are not (Rezzonico & Duffy, 2008).

Besides the metabolic link described above, many other functions have been assigned to bacterial chemical signals. For instance, Bredenbruch *et al.* (2006) have demonstrated that the *P. aeruginosa* PQS signal has iron-chelating properties. Using transcriptome analyses of the *P. aeruginosa* response to PQS, the authors showed that a large group of differentially regulated genes were involved in iron metabolism. This prompted the authors to investigate a direct link between PQS and iron homeostasis, leading to the discovery that PQS has iron-chelating activity and can form iron-containing complexes. Also, some of the known effects of PQS on *P. aeruginosa* could be attributed to its direct action as an iron chelator, supporting the notion that bacterial signals may have many currently unidentified functions in bacterial physiology (Bredenbruch *et al.*, 2006).

Another non-signalling function that has been assigned to bacterial signalling molecules is that of antimicrobials. Lantibiotics are small peptides produced by Gram-positive bacteria, and they have been studied for several decades due to their antibiotic activity (Asaduzzaman & Sonomoto, 2009). More recently, it has been found that in several cases, lantibiotics act as signalling molecules that regulate their own synthesis, much like the molecules originally described as AIs decades ago. A few examples of this involve the production of mersacidin and subtilin by *Bacillus* (Kleerebezem, 2004), nisin production by *Lactococcus lactis* (Kuipers *et al.*, 1995) and streptin production by *Streptococcus pyogenes* (Wescombe & Tagg, 2003). It is worth noting that many other well-known antibiotics have also been shown to act as signalling molecules (Goh *et al.*, 2002). At subinhibitory concentrations, antibiotics can have a substantial impact on bacterial gene expression, suggesting that they can act as signals as well as antimicrobials.

Bacterial AIs can also be used for competition between species or strains. In this case they do not act as true signalling molecules but rather as signalling inhibitors. As mentioned before, quorum sensing in *S. aureus* involves the production of a small peptide that can control gene expression in a cell-density-dependent manner (Novick & Geisinger, 2008). This AIP can control the expression of several *S. aureus* exoproducts. Ji *et al.* (1997) showed that different strains of *S. aureus* produced different peptides and that peptides from one strain could inhibit gene expression by other strains. This allowed the authors to divide *S. aureus* strains into three *agr* compatibility groups, based on the activity of their autoinducing peptides. Later on, a fourth *agr* compatibility group was described (Jarraud *et al.*, 2000). These findings raised the possibility that different *S. aureus* clones could use *agr* inhibition to compete against each other during the colonization of hosts. Indeed, specific *agr* groups can be associated with different infections caused by *S. aureus* (Jarraud *et al.*, 2000, 2002; Musser *et al.*, 1990), suggesting that quorum sensing in *S. aureus* may function not only as a mechanism to control gene expression but also as a tool used to eliminate competitors and allow more efficient host colonization.

The discovery that bacterial AIs have many functions that are not directly related to signalling has implications for the study of the evolution of quorum sensing. Explaining social behaviours through the lens of evolution has been a major challenge for evolutionary biologists, and this is also true for the study of microbial group behaviours (Diggle *et al.*, 2007a; West *et al.*, 2006). Non-signalling functions of microbial small molecules could therefore represent non-social functions from which these molecules could be borrowed during the evolution of microbial signalling. Although this hypothesis remains to be experimentally

investigated, the studies described above indicate that there may be many interesting properties of microbial signals that are yet to be recognized.

Concluding remarks and future directions

In the last few decades, much has been learned about the mechanisms used by bacteria to communicate and control virulence traits. Still, new molecules and their effects on microbial virulence continue to be discovered. It is clear that the interface between quorum sensing and bacterial virulence represents a promising area from which new, effective antivirulence drugs can emerge. The examples cited here demonstrate that inhibition of virulence through inhibition of quorum sensing is possible and somewhat practical. The challenge ahead lies in translating this knowledge into real therapeutics that could boost our nearly exhausted supply of effective antibiotics.

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