Intrinsic and extrinsic regulation of type III secretion gene expression in Pseudomonas aeruginosa

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that is particularly problematic in the healthcare setting where it is a frequent cause of pneumonia, bloodstream, and urinary tract infections. An important determinant of P. aeruginosa virulence is a type III secretion system (T3SS). T3SS-dependent intoxication is a complex process that minimally requires binding of P. aeruginosa to host cells, injection of the cytotoxic effector proteins through the host cell plasma membrane, and induction of T3SS gene expression. The latter process, referred to as contact-dependent expression, involves a well-characterized regulatory cascade that activates T3SS gene expression in response to host cell contact. Although host cell contact is a primary activating signal for T3SS gene expression, the involvement of multiple membrane-bound regulatory systems indicates that additional environmental signals also play a role in controlling expression of the T3SS. These regulatory systems coordinate T3SS gene expression with many other cellular activities including motility, mucoidy, polysaccharide production, and biofilm formation. The signals to which the organism responds are poorly understood but many seem to be coupled to the metabolic state of the cell and integrated within a master circuit that assimilates informational signals from endogenous and exogenous sources. Herein we review progress toward unraveling this complex circuitry, provide analysis of the current knowledge gaps, and highlight potential areas for future studies. Complete understanding of the regulatory networks that control T3SS gene expression will maximize opportunities for the development of strategies to treat P. aeruginosa infections.

Keywords: Pseudomonas aeruginosa, type III secretion, injectisome, gene regulation, ExsA, cAMP, RsmA, Vfr

P. aeruginosa is an opportunistic pathogen that is particularly problematic in the healthcare setting where it is a frequent cause of pneumonia, bloodstream, and urinary tract infections. An important determinant of P. aeruginosa virulence is a type III secretion system (T3SS). T3SS-dependent intoxication is a complex process that minimally requires binding of P. aeruginosa to host cells, injection of the cytotoxic effector proteins through the host cell plasma membrane, and induction of T3SS gene expression. The latter process, referred to as contact-dependent expression, involves a well-characterized regulatory cascade that activates T3SS gene expression in response to host cell contact. Although host cell contact is a primary activating signal for T3SS gene expression, the involvement of multiple membrane-bound regulatory systems indicates that additional environmental signals also play a role in controlling expression of the T3SS. These regulatory systems coordinate T3SS gene expression with many other cellular activities including motility, mucoidy, polysaccharide production, and biofilm formation. The signals to which the organism responds are poorly understood but many seem to be coupled to the metabolic state of the cell and integrated within a master circuit that assimilates informational signals from endogenous and exogenous sources. Herein we review progress toward unraveling this complex circuitry, provide analysis of the current knowledge gaps, and highlight potential areas for future studies. Complete understanding of the regulatory networks that control T3SS gene expression will maximize opportunities for the development of strategies to treat P. aeruginosa infections.

Keywords: Pseudomonas aeruginosa, type III secretion, injectisome, gene regulation, ExsA, cAMP, RsmA, Vfr

INTRINSIC REGULATION OF T3SS GENE EXPRESSION

ExsA is the central regulator of T3SS gene expression

The P. aeruginosa T3SS regulon consists of ~40 genes organized within 10 transcriptional units that encode the secretion and translocation machinery, intrinsic regulators of T3SS gene expression

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(ExsA, ExsD, ExsC, and ExsE), chaperones, and effectors (Frank, 1997). The central regulator of the T3SS regulon is ExsA, a member of the AraC/XylS family of transcriptional activators (Yahr and Frank, 1994; Hovey and Frank, 1995; Yahr et al., 1995). ExsA autoregulates its own expression through the P_{exoC} promoter and is encoded as the last gene of the exsCEBA polycistronic mRNA (Yahr and Frank, 1994). An alignment of the 10 ExsA-dependent promoters has identified a consensus binding site (tAaAAAnwnMyGrCynnnmYTGayAk) with three areas of high sequence conservation: an adenine-rich region, a GxC sequence centered at the -45 position with respect to the transcriptional start site, and a TGxxA sequence located near the -35 position (Figure 1; Hovey and Frank, 1995; Brutinel et al., 2008). Mutagenesis studies have confirmed that each of these elements is required for ExsA binding and activation of the P_{exoC} promoter (Brutinel et al., 2008).

Typical of most AraC/XylS family members, ExsA has an amino-terminal domain (NTD) involved in self-association and regulatory functions (discussed further below), and a carboxy-terminal domain (CTD) that contains two helix-turn-helix DNA-binding motifs (Brutinel et al., 2009). In electrophoretic mobility shift assays (EMSA) purified ExsA binds to the P_{exoC}, P_{exoD}, and P_{exoE} promoters with apparent equilibrium constants in the low (1–5) nM range (Brutinel et al., 2008). Two distinct ExsA-promoter complexes are observed: a higher mobility complex representing one ExsA monomer bound to the promoter probe and a lower mobility complex containing two bound ExsA monomers (Brutinel et al., 2008). Mutagenesis and footprinting studies indicate that this binding arrangement represents two adjacent binding sites for monomeric ExsA with binding site 1 overlapping the conserved GxC and TGxxA sequences, and binding site 2 located upstream of site 1 (Figure 1). Whereas binding of ExsA to site 1 of the P_{exoC} promoter is required for occupation of site 2, deletion of binding site 2 has no effect on occupation of site 1 (Brutinel et al., 2008). In addition, occupation of binding site 2 is dependent upon monomer–monomer interactions mediated by the amino-terminal domain of ExsA (Brutinel et al., 2009). These combined data are supportive of a monomer assembly model whereby an ExsA monomer bound to site 1 recruits a second ExsA monomer to binding site 2. The apparent lack of nucleotide sequence conservation between ExsA binding sites 1 and 2, however, raises the possibility that monomer–monomer

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**FIGURE 1** | Sequence alignment of ExsA-dependent promoters and working model for promoter recognition and recruitment of σ^{70}-RNAP by ExsA. Nucleotides in bold are highly conserved in all ExsA-dependent promoters and have been shown through mutagenesis and DNA-binding studies to be important for ExsA-dependent activation. The boxed sequences represent the -10 promoter recognition site. Predicted points of interaction for the helix-turn-helix (HTH) DNA-binding motifs of each ExsA monomer are indicated. Studies have shown that ExsA recruits σ^{70}-RNAP through interactions mediated by region 4.2 of σ^{70}. The ExsA consensus binding sequence is indicated at the bottom of the diagram.
interactions are the primary determinant for occupation of site 2. While genetic studies are suggestive of cooperative binding to the P_70-promoter, the Hill coefficients derived from EMSA experiments are supportive of cooperative binding only at the P_70-promoter (1.9 ± 0.2; Brutinel et al., 2008). This discrepancy may reflect a fundamental difference in the binding of ExsA to the P_70-promoter and is supported by the observation that ExsA elicits a significant bend (78°) upon binding to the P_70-promoter that correlates with increased promoter activity and cooperative binding interactions (Brutinel et al., 2008).

ExsA-dependent promoters possess -35 and -10 sequence elements similar to those of σ^70-dependent promoters with one significant difference (Brutinel et al., 2008; Figure 1). Unlike canonical σ^70-dependent promoters where the -35 and -10 elements are separated by ~17 bp, the same regions in ExsA-dependent promoters are separated by 21 or 22 bp. In vitro transcription and primer extension assays indicate that the promoters are indeed σ^70-dependent and that the -10 regions are functional recognition determinants for σ^70 (Vakulskas et al., 2009). The apparent -35 elements, however, are not required for recognition by σ^70 but rather serve as determinants for ExsA binding (Vakulskas et al., 2010). ExsA activates transcription by compensating for the lack of functional -35 sites primarily through recruitment of RNA polymerase-σ^70 holoenzyme to the promoter but also has a small effect on isomerization to an open complex (Vakulskas et al., 2009). Like many AraC/XylS family members, ExsA interacts with region 4.2 of σ^70 to recruit RNAP-σ^70 to the promoter (Vakulskas et al., 2010). Somewhat atypical for an AraC/XylS family member, however, is that interactions with the α-subunit of RNAP are not required for ExsA-dependent activation. Although occupation of binding sites 1 and 2 on the promoter is required for ExsA-dependent transcription, it is unclear whether one or both monomers interact with RNAP-σ^70 to facilitate recruitment. The proximity of binding site 1 to the expected location for a -35 region, and the fact that ExsA compensates for the lack of a functional -35 region, is consistent with a model in which ExsA bound to site 1 interacts with region 4.2 of σ^70 and ExsA bound to site 2 stabilizes ExsA bound to site 1 and/or the ExsA-RNAP-σ^70 complex.

REGULATION OF EXSA ACTIVITY BY THE EXSADCE REGULATORY CASCADE

Type III secretion systems have been described as contact-dependent secretion systems because translocation of the effector proteins requires intimate contact between the pathogen and the host, and because host cell contact induces T3SS gene expression (Hayes et al., 2010). Growth of P. aeruginosa under Ca^2+ -limiting conditions, or in the presence of serum, mimics the host-contact signal and is commonly used in the laboratory as a surrogate for the host-contact signal (Frank, 1997; Vallis et al., 1999; Kim et al., 2005; Urbanowski et al., 2007). Low Ca^2+, serum, and host cell contact are each thought to convert the injectosome to a secretion-competent state through a poorly defined mechanism (Vallis et al., 1999; McCaw et al., 2002). ExsA activity is intimately coupled to secretion by a partner-switching mechanism involving three additional proteins; ExsC, ExsD, and ExsE (Brutinel and Yahr, 2008). Both ExsC and ExsD have two potential binding partners. ExsD is an anti-activator that inhibits T3SS gene expression by disrupting both the self-association and DNA-binding activities of ExsA (Brutinel et al., 2010). ExsC functions as an anti-anti-activator and forms a 2:2 stoichiometric complex with ExsD (Dasgupta et al., 2004; Lykken et al., 2006; Zheng et al., 2007). ExsC is also a T3SS chaperone and forms a 2:1 complex with ExsE (Zheng et al., 2007). Finally, ExsE is a secreted regulator that prevents ExsC from associating with ExsD (Rietsch et al., 2005; Urbanowski et al., 2005). The current working model is that ExsA-dependent transcription is inactive under non-permissive conditions (i.e., high Ca^2+) because the binding equilibria favor formation of the inhibitory ExsD•ExsA and ExsC•ExsE complexes. In contrast, inducing conditions trigger secretion and/or translocation of ExsE (Urbanowski et al., 2007). The resulting decrease in the intracellular concentration of ExsE favors formation of the ExsD•ExsC complex (i.e., partner switching), thereby releasing ExsA to activate transcription. An important feature of the regulatory cascade is that minor changes in the levels of ExsC, ExsE, ExsA, or ExsD (as little as threefold) can have profound effects on T3SS gene expression (Dasgupta et al., 2004).

The partner-switching mechanism predicts that the binding affinities of ExsE, ExsC, ExsD, and ExsA are, in order of strongest affinity, ExsC•ExsE > ExsC•ExsD > ExsD•ExsA. While the binding affinity of the ExsD–ExsA complex has not been determined, the binding constants (Kd) for the ExsC•ExsE and ExsC•ExsD complexes are 1 and 18 nM, respectively (Zheng et al., 2007). Recent analyses of the ExsE•ExsC and ExsD crystal structures provide a basis for the differential binding of ExsC to ExsE and ExsD (Bernhards et al., 2009; Vogelaar et al., 2010). The ExsE monomer contains two ExsC-binding domains located in the amino- and carboxy-terminal portions of the protein and each domain contains an Arg-X-Val-X-Arg motif and a partially overlapping β-motif. In the co-complex the unstructured ExsE monomer wraps around the ExsC homodimer and each Arg-X-Val-X-Arg motif interacts with one of the ExsC monomers, thereby accounting for the 2:1 binding stoichiometry of the ExsC•ExsE complex. Interestingly, the amino-terminal region of ExsD also has an Arg-X-Val-X-Arg motif, a β-motif, and shares 22.5% sequence identity with the amino-terminus of ExsE. Whereas the entire ExsE protein (i.e., both Arg-X-Val-X-Arg motifs) is necessary for formation of the ExsC•ExsE complex, the ExsD amino-terminal region alone is sufficient to bind ExsC. This observation suggests that each ExsD monomer binds to one-half of the ExsC dimer and this likely accounts for the 2:2 binding stoichiometry of the ExsC•ExsD complex. Furthermore, the presence of a common ExsC-binding motif in ExsE and ExsD is consistent with the observation that binding of ExsE and ExsD to ExsC are mutually exclusive (Vogelaar et al., 2010).

ExsD is a dynamic protein capable of forming a homo–trimeric complex in solution, a 1:1 stoichiometric complex with ExsA, and a 2:2 stoichiometric complex with ExsC (Zheng et al., 2007; Bernhards et al., 2009; Thibault et al., 2009). Curiously, the ExsD crystal structure revealed that the amino-terminus of ExsD has structural similarity to the DNA-binding domain of the KorB transcriptional repressor (Bernhards et al., 2009). The predicted DNA-binding interface, however, is buried within the trimeric structure suggesting that dissociation of the trimer might be required for ExsD to exhibit DNA-binding activity. This property might account for the observation that trimeric ExsD lacks
DNA-binding activity (Bernhards et al., 2009). Future studies will be required to determine whether monomeric and/or dimeric ExsD has DNA-binding activity and, if so, whether such activity is of biological significance.

Studies of the ExsD•ExsA interaction have relied upon co-expression of the proteins in vivo and subsequent purification as an ExsD•ExsA complex. Consistent with the functional role of ExsD as an anti-activator, the purified ExsA•ExsD complex lacks DNA-binding activity (Thibault et al., 2009; Brutinel et al., 2010). ExsA dissociated from the ExsD•ExsA complex through ExsC addition, however, is able to bind to DNA, indicating that ExsD does not irreversibly inactivate ExsA (Brutinel et al., 2010). The initial and rapid transcriptional response to inducing signals, therefore, likely reflects dissociation of existing ExsD•ExsA complexes by ExsC.

The response to non-inducing signals (i.e., high Ca\textsuperscript{2+}) appears more complicated because neither purified trimeric ExsD, nor ExsD dissociated from the ExsC•ExsD complex, is capable of binding to ExsA in vitro (Thibault et al., 2009; Brutinel et al., 2010). These data, when combined with the observation that ExsD•ExsA complex formation is dependent upon concurrent expression of the proteins in vivo, suggest that complex formation may involve a folding intermediate of either ExsD or ExsA. ExsD dissociated from the ExsC•ExsD complex, therefore, does not contribute to the inhibition of ExsA-dependent transcription. Furthermore, the fact that trimeric ExsD is unable to bind to ExsA suggests two potential pathways for irreversible inactivation of ExsD: homo−trimerization and ExsC•ExsD complex formation. The response to inhibiting signals (i.e., high Ca\textsuperscript{2+}) exhibits a lag period (Diaz and Yahrl, unpublished) that may reflect a requisite decay of the existing free ExsA and a requirement for de novo synthesis of ExsD to sequester the newly synthesized ExsA.

ExsD inhibits both the self-association and DNA-binding properties of ExsA (Thibault et al., 2009; Brutinel et al., 2010). Since the amino-terminal domain of ExsA mediates self-association and is sufficient for complex formation with ExsD, ExsD likely inhibits ExsA self-association through steric hindrance. Less clear is how ExsD inhibits the carboxy-terminal DNA-binding domain when bound to the amino-terminus of ExsA. Resolution of this question will require structural studies to determine whether bound ExsD elicits a conformational change to the DNA-binding domain of ExsA or physically occludes one or both of the helix-turn-helix DNA-binding domains.

PHYSIOLOGICAL IMPLICATIONS OF THE ExsADCE REGULATORY CASCADE

The T3SS functions as a primary defense mechanism against phagocytosis and must be rapidly activated following contact with a phagocyte. Prior to host cell contact, P. aeruginosa cells already express low levels of preexisting injectisomes (Rietsch and Meakalanos, 2006) and effectors that initiate the defense response. The ExsADCE regulatory cascade functions to increase the magnitude of the response by rapidly inducing expression of the injectisome components and effectors. While upregulation of T3SS gene expression would be important at the level of individual cells, in the context of an infection this requirement may not extend to the population as a whole. Single cell expression studies have demonstrated that T3SS gene expression exhibits bistability whereby only a fraction of the cells demonstrate increased expression in response to inducing signals (both low Ca\textsuperscript{2+} and host cell contact; Horner et al., 2000; Rietsch et al., 2004; Urbanowski et al., 2007). Bistability is a stochastic process generally reflecting noise in the expression of a master regulator (Dubnau and Losick, 2006). Since minor changes in the expression levels of ExsA, ExsD, ExsC, or ExsE have profound effects on T3SS gene expression, bistable expression of the T3SS is likely mediated through the ExsADCE regulatory cascade. The biological significance of bistability is not entirely clear. Bistability might reflect energetic considerations whereby only a subpopulation of T3SS-expressing P. aeruginosa are necessary to neutralize phagocytic clearance or elicit T3SS-dependent cytotoxicity during later stages of colonization. In this context, cells lacking T3SS gene expression could be likened to the social cheats described in quorum sensing responses (Sandoz et al., 2007). Alternatively, bistability may reflect a state of heightened activation that allows a fraction of the cells to respond rapidly to predators. Finally, there may be a selective advantage for cells that lack T3SS gene expression such as avoidance of antibody responses directed against the injectisome.

Whereas the ExsADCE system allows for rapid induction of T3SS gene expression, there is a notable lag period in down-regulation following exposure to high Ca\textsuperscript{2+} conditions (as described above) and this lag period likely occurs following the loss of host cell contact as well. This regulatory feature may provide for a slow taper in T3SS gene expression which, during the interim period between full induction and the return to basal expression levels, serves as a safeguard against subsequent encounters with phagocytes.

EXTRINSIC REGULATION OF T3SS GENE EXPRESSION

The ExsADCE regulatory pathway is central to T3SS gene regulation but does not operate in isolation. As described in a previous review (Yahr and Wolfgang, 2006), many extrinsic regulatory pathways involving no less than 30 gene products influence T3SS gene expression. In nearly every case, the mechanisms by which they exert control over T3SS gene expression is poorly understood. One of the future challenges facing the field is to account for regulation by each of these pathways and identify the signals to which they respond. Despite significant knowledge gaps, molecular details are beginning to emerge for some of these systems.

GLOBAL REGULATION OF T3SS GENE EXPRESSION BY Vfr

Virulence factor regulator (Vfr) is a cAMP-dependent regulator of transcription that was first identified as an activator of extracellular protease and exotoxin A expression (West et al., 1994). Now appreciated as a global regulator of virulence gene expression, the Vfr regulon consists of ∼200 genes including type IV pili, a type II secretion system, quorum sensing, and the T3SS (Wolfgang et al., 2003). In addition to Vfr, the system consists of two adenylyl cyclases (CyAa and CyAB) and a CAMP phosphodiesterase (Figure 2; CpdA; Wolfgang et al., 2003; Fuchs et al., 2010). Both high osmolality (NaCl) and low Ca\textsuperscript{2+} stimulate CAMP production (Wolfgang et al., 2003), and CAMP homeostasis is tightly controlled by CpdA (Fuchs et al., 2010). Although a Vfr consensus binding sequence has been determined (Kanack et al., 2006), the mechanism by which Vfr controls T3SS gene expression remains unknown and represents a significant milestone for future studies.
INVERSE REGULATION OF MUCOIDY AND T3SS GENE EXPRESSION

The role of the *P. aeruginosa* T3SS is well established within the context of acute infections where it contributes to tissue damage, sepsis, and mortality (Engel and Balachandran, 2009). *P. aeruginosa* is also the most common cause of chronic airway infection in individuals with cystic fibrosis (CF). CF infections are characterized by biofilm growth, slow progression, minimal tissue damage, and restriction to the airways (Gomez and Prince, 2007). The mechanistic basis for the striking difference in the progression of acute (non-CF) and chronic CF infections is not well understood. One contributing factor is that many *P. aeruginosa* virulence determinants are differentially expressed during non-CF and CF infections. Although CF and non-CF patients are initially colonized with nearly identical strains of *P. aeruginosa*, the majority of chronic CF isolates are mucoid and lack T3SS gene expression (Alonso et al., 1999; Feltman et al., 2001; Allmond et al., 2004). Mucoid conversion results from overproduction of the alginate polysaccharide and is associated with a decline in lung function and clinical prognosis (Pedersen et al., 1992; Li et al., 2005). Mucoid conversion and the loss of T3SS gene expression are now known to be linked events involving *mucA* mutation (Wu et al., 2004; Jones et al., 2010).

Alginate biosynthesis is regulated by the AlgU alternative sigma factor and the membrane-bound MucA anti-sigma factor (Wozniak and Ohman, 1994; Govan and Deretic, 1996). MucA normally sequesters AlgU to prevent expression of the AlgU regulon (Figure 2; Schurr et al., 1996; Mathee et al., 1997). MucA is (indirectly) responsive to a variety of environmental signals (primarily stress signals) resulting in the release of AlgU (Wood and Ohman, 2009). Liberated AlgU activates many genes including the AlgZR two-component regulatory system and the alginate biosynthetic genes (Wozniak and Ohman, 1994). While environmental signals represent one mechanism for activation of the AlgU regulon, mucoid conversion in CF isolates usually results from nonsense mutations in *mucA* (Boucher et al., 1997; Mathee et al., 1997). Jin and colleagues were the first to observe that *mucA* mutants are defective for T3SS gene expression and found the inhibition to be mediated through the AlgZR two-component system (Wu et al., 2004). Wolfgang and colleagues later noted that *vfr* and *mucA* mutants share several common phenotypes including reduced expression of the T3SS, T2SS, exotoxin A, and type IV pili, and subsequently confirmed that *mucA* mutants are defective for *vfr* transcription (Figure 2; Jones et al., 2010). The lack of T3SS gene expression in *mucA* mutants, therefore, reflects a defect in cAMP/Vfr signaling. Coincident mucoid conversion and loss of cAMP/Vfr signaling suggests that the selective advantage of *mucA* mutants in the CF airways may extend beyond mucoidy itself (Rau et al., 2010). Finally, the observation that *algZR* mutants have increased T3SS gene expression suggests that AlgZR modulates expression of the T3SS even in the presence of functional MucA (Jones et al., 2010). Since the MucA/AlgU system controls a generalized stress response, an interesting question for the future is whether stress signals encountered within the host modulate T3SS gene expression through MucA/AlgU.

T3SS GENE EXPRESSION AND BIOFILM FORMATION

Like alginate production, *P. aeruginosa* biofilms are thought to contribute to the chronic nature of CF infections (Singh et al., 2000). Biofilm formation is a complex adaptation subject to multiple layers of regulation (Harmsen et al., 2010). One regulatory mechanism involves the RetS and LadS sensor kinases. Whereas a retS (regulator...
of exopolysaccharide and type III secretion) mutant is defective for T3SS gene expression and displays enhanced biofilm formation, a ladS mutant has the reciprocal phenotypes (Goodman et al., 2004; Ventre et al., 2006). Mechanistically, RetS and LadS mediate opposite effects on activation of the GacAS system (Figure 2). The GacAS system regulates biofilm formation through control of the Pel and Psl polysaccharides, key components of the biofilm matrix (Ryder et al., 2007). Psl biosynthesis is controlled at the post-transcriptional level by RsmA, an RNA-binding protein that binds directly to the 5′ UTR region of the pslA mRNA to inhibit translational initiation (Irie et al., 2010). In response to unknown signals, GacA activates transcription of two small non-coding regulatory RNAs (RsmY and RsmZ). RsmY and RsmZ each have ~4–6 binding sites for RsmA and function by sequestering free RsmA from target mRNAs (Lapouge et al., 2008). Decreased levels of free RsmA, therefore, are required for Psl expression. In contrast, T3SS gene expression is dependent upon increased levels of free RsmA (Goodman et al., 2004; Mulcahy et al., 2006). The mechanism by which RsmA promotes T3SS gene expression is poorly understood. RsmA is a member of the CsrA family of proteins first described in E. coli (Romeo et al., 1993). Although CsrA family members usually prevent translation by binding to or near the Shine–Dalgarno (SD) sequence of target mRNAs (Lapouge et al., 2008), direct positive regulation by CsrA has also been reported and involves mRNA stabilization (Wei et al., 2001). This leaves several scenarios to account for positive regulation of T3SS gene expression by RsmA. RsmA may function directly at the level of intrinsic regulation by promoting expression of a positive (ExsA or ExsC) acting factor or inhibiting expression of negative (ExsD or ExsE) regulator. Alternatively, RsmA may function indirectly by modulating an extrinsic regulatory pathway that controls the T3SS. With respect to the latter possibility it is noteworthy that the RsmA regulon is extensive and includes rhamnolipid production, lipase production, swarming motility, quorum sensing, lectin, pyocyanin, hydrogen cyanide, and the type VI secretion system (Pessi et al., 2001; Heurlier et al., 2004; Brencic and Lory, 2009). The possibility exists, therefore, that the regulatory activity of RsmA toward T3SS gene expression results from pleiotropic effects that may be difficult to define.

The observation of inverse regulation by RetS and LadS implied that T3SS gene expression and biofilm formation were mutually exclusive events. It is now becoming apparent, however, that such a model is an over-simplification and that the T3SS is possible during biofilm growth (Mikkelsen et al., 2009).

T3SS GENE EXPRESSION AND MOTILITY

All three forms of P. aeruginosa motility (type IV pilus-based twitching, swarming, and flagellar-based swimming,) are coordinated (either positively or negatively) with T3SS gene expression. As described above, type IV pili and T3SS gene expression are positively regulated by Vfr (Jones et al., 2010). The biological significance of this relationship reflects the adherence properties provided by type IV pili, which function as the primary adhesin for host-contact-dependent expression of the T3SS (Sundin et al., 2002). T3SS gene expression is also positively correlated with swarming motility, which might be advantageous during colonization of mucosal surfaces (Overhage et al., 2008). In contrast, flagellar gene expression is inversely regulated with T3SS gene expression. Whereas a flIC mutant is deregulated for T3SS gene expression (three- to ninefold) and displays increased cytotoxicity toward a macrophage cell line, ExsA over-expression reduces motility and expression of a flia transcriptional reporter (Soscia et al., 2007). The reciprocal nature of T3SS and flagellar gene expression is interesting to contemplate in the context of inflammasome activation. Several studies suggest that flagellin (FlIC) translocated into macrophages by the P. aeruginosa T3SS activates the inflammasome leading to a proinflammatory response (Franchi et al., 2007; Galle et al., 2007; Sutterwala et al., 2007; Miao et al., 2008). Despite experimental evidence of inflammasome activation ex vivo, it is unclear whether this occurs in vivo because the ExoS and ExoU effectors both block FlIC-dependent inflammasome activation. The additional finding that FlIC expression is inversely regulated with T3SS gene expression suggests that inflammasome activation is either relatively unimportant, does not normally occur during infections, or occurs under conditions that remain to be defined.

ENVIRONMENTAL SIGNALS THAT MODULATE T3SS GENE EXPRESSION

Ca2+-limitation and host cell contact are well-defined signals that activate transcription through the control of T3SS secretory activity. Many additional signals, arising from both endogenous and exogenous sources, also influence T3SS gene expression and are summarized in Table 1. Evident is the fact that these signals are diverse with stress and metabolic signals representing the largest class. Less obvious is that the mechanisms by which these signals influence T3SS gene expression are poorly understood. In fact, besides ExsA only one additional transcriptional factor (PsrA) is known to directly regulate the T3SS. PsrA binds the PpsrA promoter to positively control transcription of the exsCBA operon (Shen et al., 2006). Interestingly, the DNA-binding activity of PsrA is directly inhibited by long chain fatty acids. Indeed T3SS gene expression is reduced in the presence of long chain fatty acids, although the biological significance of this observation is unclear (Kang et al., 2009). The involvement of PsrA is discussed here to illustrate the point that cross-talk between different regulatory systems coordinates T3SS gene expression with diverse cellular functions. In addition to the T3SS, PsrA regulates a diverse set of genes including type IV pili (a potential link to Vfr), the psl biosynthesis operon (a potential link to RsmA), the stationary phase sigma factor rpoS (a potential link to quorum sensing) and a variety of genes involved in metabolic and energetic metabolism. Furthermore, psrA mutants are defective for swarming motility, biofilm formation, and resistance to some antibiotics. In just this one example, therefore, one begins to appreciate the level of complexity involved in controlling T3SS gene expression. Despite the involvement of numerous signals and gene products, it seems likely that reductionist approaches will ultimately reveal that only a minority (i.e., PsrA, Vfr, RsmA) of the genes implicated in regulation of the T3SS directly participate in the control of T3SS gene expression. Future explorations aimed as examining whether signals and gene products exert their regulatory effects at the level of ExsADCE or through upstream regulatory pathways are required to fully integrate the regulatory pathways leading to T3SS gene expression.
Table 1 | Environmental signals that influence T3SS gene expression.

| Signal                          | Effect on T3SS | Implicated genes                  | Reference                                      |
|---------------------------------|----------------|----------------------------------|------------------------------------------------|
| **ACTIVATION OF SECRETION**     |                |                                  |                                                |
| Host cell contact               | +              | exsADCE                          | Vallis et al. (1999), Urbanowski et al. (2007) |
| Low calcium                     | +              | exsADCE                          | Frank (1997)                                   |
| Serum/albumin                   | +              | exsADCE                          | Vallis et al. (1999), Kim et al. (2005)        |
| **ENVIRONMENTAL STRESS**        |                |                                  |                                                |
| DNA damage                      | −              | recA, ptrB, ptrN, ptrR           | Wu and Jin (2005)                              |
| Hyperosmotic stress             | +              | cAMP/Vfr                         | Aspedon et al. (2006), Rietsch and Mekalanos (2006) |
| Copper stress                   | −              | ptrA                             | Ha et al. (2004)                                |
| **METABOLIC SIGNALS/STRESSES**  |                |                                  |                                                |
| Pyruvate imbalance              | −              | aceA, aceB                        | Dacheux et al. (2002)                          |
| Unknown                         | +              | spuE                             | Zhou et al. (2007)                             |
| Spermidine/spermine             | +              | tpaA, leuC                        | Shen et al. (2008)                             |
| Tryptophan metabolites          | −              | prsA, rpoS                        | Kang et al. (2009)                             |
| Long chain fatty acids          | −              | unknown                          | Tralau et al. (2007)                           |
| Mucin                           | ±              | unknown                          | Singh et al. (2010)                            |
| PQS                             | −              | pqsR, mvfR                        |                                                 |
| **ANAEROBIOSES**                |                |                                  |                                                |
| NO                              | +              | nirS, narGH                       | Van Alst et al. (2009)                         |
| **ANTIBIOTIC EXPOSURE**         |                |                                  |                                                |
| Azithromycin                    | +              | mexCD-oprJ, ptrA                  | Gillis et al. (2005)                           |
| Chloramphenicol                 | −              | mexEF                            | Linares et al. (2005)                          |
| Tetracycline                    | +              | unknown                          | Linares et al. (2006)                          |
| Antimicrobial peptides          | −              | prsA                             | Gooderham et al. (2008)                        |
| Quorum sensing                  | −              | rpoS                             | Hogardt et al. (2004), Bleves et al. (2005)    |

**CHALLENGES FOR THE FUTURE**

The literature contains a wealth of information regarding genes and conditions that contribute to T3SS gene expression in *P. aeruginosa*. The challenge ahead lies in assimilating this information into a unified regulatory network supportive of all prior and future observations. In absolute terms this will undoubtedly prove impossible owing to differences in mutant phenotypes, strain backgrounds, growth conditions, and experimental findings. In analyzing the primary literature consideration of the following points is important to keep in mind: (i) Nearly 20 microarray studies have noted alterations in T3SS gene expression. Most of these studies were conducted under non-permissive conditions for T3SS gene expression (i.e., high Ca²⁺) making it difficult to ascertain whether the observed changes represent minor alterations to the basal level of T3SS transcription or more significant regulatory effects. (ii) To the best of our knowledge, plasmid-expressed ExsA is able to restore transcription to all mutants identified as defective for T3SS transcription. Reports of regulatory effects occurring upstream of ExsA, therefore, must be interpreted with caution since ExsA over-expression can easily obscure the activity of and/or requirement for other regulatory systems. (iii) Although most studies of T3SS gene expression have relied upon Ca²⁺ chelation as the inducing signal, it is unlikely that Ca²⁺ chelation and host cell contact are entirely analogous signals. Future studies are required to fully define the physiologically relevant signals encountered in vivo and determine how they modulate or fine-tune T3SS gene expression. (iv) The induction of T3SS gene expression is minimally dependent upon a functional injectisome, the ExsADCE cascade, and the Vfr and RsmA global regulatory systems and is an important consideration in that regulatory phenotypes that alter T3SS gene expression might result from pleiotropic effects without actually contributing to the regulatory process per se.

Therapeutic targeting of the T3SS is an achievable goal with potential intervention points including the inhibition of assembly, secretion, translocation, and T3SS gene expression. Since ExsA-dependent transcription is dependent upon an intact secretion and translocation machinery, each class of inhibitor offers the added benefit of reduced T3SS gene expression. Most efforts to date have logically targeted the highly conserved secretion/translocation machinery with the hope of identifying inhibitors that possess activity against multiple pathogens. A limitation of this approach lies in identification of the inhibitor target since each of the ~25 constituents of the secretion/translocation machinery as well as their interaction interfaces represent potential target sites. Direct targeting of transcription offers the advantage that many promising targets already exist including the ExsADCE regulatory cascade, and the cAMP/Vfr, MucA/AlgU, and GacA/RsmA global regulatory pathways, and in fact inhibitors of ExsA-dependent transcription have already been identified (Grier et al., 2010). Time will tell whether such inhibitors can deliver on the promise of controlling *P. aeruginosa* infections.

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