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MINIREVIEW

Fitting Pieces into the Puzzle of *Pseudomonas aeruginosa* Type III Secretion System Gene Expression

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ABSTRACT Type III secretion systems (T3SS) are widely distributed in Gram-negative microorganisms and critical for host-pathogen and host-symbiont interactions with plants and animals. Central features of the T3SS are a highly conserved set of secretion and translocation genes and contact dependence wherein host-pathogen interactions trigger effector protein delivery and serve as an inducing signal for T3SS gene expression. In addition to these conserved features, there are pathogen-specific properties that include a unique repertoire of effector genes and mechanisms to control T3SS gene expression. The *Pseudomonas aeruginosa* T3SS serves as a model system to understand transcriptional and posttranscriptional mechanisms involved in the control of T3SS gene expression. The central regulatory feature is a partner-switching system that controls the DNA-binding activity of ExsA, the primary regulator of T3SS gene expression. Superimposed upon the partner-switching mechanism are cyclic AMP and cyclic di-GMP signaling systems, two-component systems, global regulators, and RNA-binding proteins that have positive and negative effects on ExsA transcription and/or synthesis. In the present review, we discuss advances in our understanding of how these regulatory systems orchestrate the activation of T3SS gene expression in the context of acute infections and repression of the T3SS as *P. aeruginosa* adapts to and colonizes the cystic fibrosis airways.

KEYWORDS *Pseudomonas aeruginosa*, type III secretion, RsmA, ExsA, DeaD, Vfr

Pseudomonas aeruginosa is an opportunistic Gram-negative pathogen capable of causing a variety of infections in humans. Known risk factors include burn wounds, corneal scratches, catheter and ventilator usage, and cystic fibrosis (1). The virulence properties of *P. aeruginosa* are multifactorial and comprise adherence factors, biofilm formation, antibiotic resistance, and secreted toxins (1). One critical virulence determinant is the type III secretion system (T3SS). The T3SS is embedded in the inner membrane and used to assemble an injectisome. The injectisome is an ~25-protein complex that spans the cell envelope and functions as a molecular syringe to translocate effector proteins into eukaryotic target cells (2). The classic effectors are ExoS, ExoT, ExoU, and ExoY (2). ExoS-secreting strains cause delayed apoptotic-like cell death, while ExoU-secreting strains cause rapid and robust host cell lysis (3, 4). Additional effectors are now appreciated and include the flagellar filament protein (FliC) (5–8), nuclear diphosphate kinase (9), and PemA/PemB (10). The translocation pore itself is sufficient to induce K^+ efflux, dephosphorylate and deacetylate histone H3, and cause host cell death (11–16). The combined activities of the translocated effectors protect *P. aeruginosa* from phagocytic and inflammatory responses, are cytotoxic, and promote systemic dissemination. Strains defective for T3SS gene expression/function are severely attenuated for virulence in burn wound, pneumonia, neutropenic, and corneal infection models (13, 17–22).

Expression of the T3SS is tightly controlled and induced in response to a number of environmental signals, including low concentrations (micromolar) of extracellular Ca^{2+},

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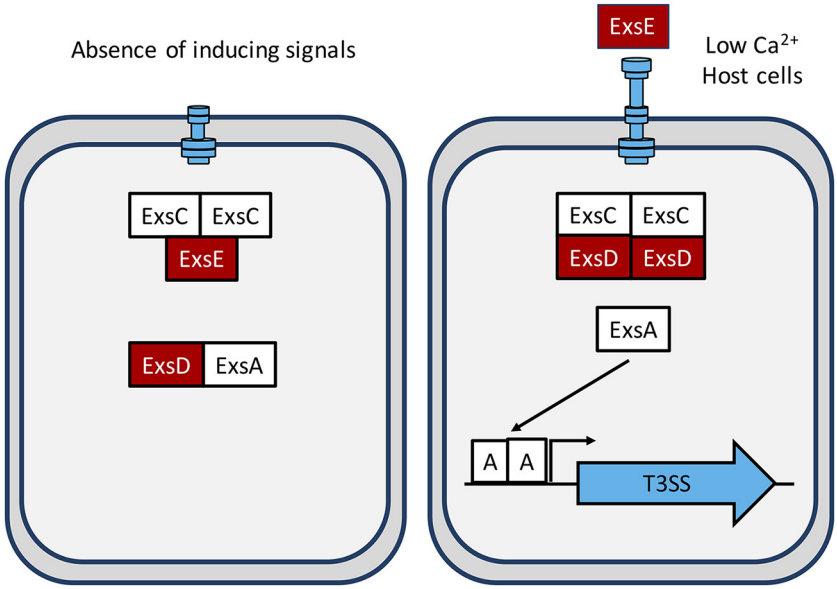

FIG 1 The T3SS partner-switching mechanism that controls the DNA-binding activity of ExsA. Negative regulators are in red, and positive regulators are in white.

serum albumin/casein, and host cell contact (23–25). Primary control of T3SS gene expression is through direct activation of transcription by ExsA, an AraC family transcription factor. Positioned upstream of ExsA is a complex regulatory network involving cyclic AMP and cyclic di-GMP signaling systems, two-component systems, global regulators, and RNA-binding proteins. In this review, we highlight the emerging theme that many of the upstream regulatory events, either directly or indirectly, control *exsA* transcription and/or translation.

ExsA AND CONTROL OF ExsA DNA-BINDING ACTIVITY

ExsA is the master regulator of T3SS gene expression. The *P. aeruginosa* T3SS regulon consists of ~40 genes encoding regulatory functions, the secretion and translocation machinery, effectors, and effector-specific chaperones (23). Most of the genes are organized into five operons and clustered in the genome at a common location. The effector genes and their associated chaperones are scattered throughout the chromosome. All of the known T3SS genes are activated by the master regulator ExsA, a member of the AraC/XylS family of transcription factors (17, 26–30). The ExsA consensus binding site is AaAAAnwnMygrCynnnmYTGayAk, centered ~45 bp upstream of the transcription start site for each of the 10 ExsA-dependent promoters (28, 31). For a more thorough review of ExsA DNA-binding properties and the mechanism of transcription activation, see the paper by Diaz et al. (32).

Control of ExsA activity by a partner-switching mechanism. In the absence of inducing signals (low Ca^{2+}, serum, and host cell contact), the injectisome is expressed at a low basal level and exists in a quiescent state (33, 34). Both of those features are critical because the injectisome is the sensor of inducing signals and responds by converting to a secretion-competent state through a poorly defined mechanism (33). Secretion competency indirectly activates T3SS gene expression through a partner-switching mechanism. The components of the partner-switching mechanism are the activator ExsA, the antiactivator ExsD, the antiantiactivator ExsC, and the secreted/translocated ExsE protein (Fig. 1). In the absence of inducing signals, the secreted substrate ExsE remains cytoplasmic in a 1:2 complex with ExsC (35), and ExsA is sequestered by ExsD in a 1:1 complex (36). Inducing signals lead to the secretion/translocation of ExsE through the injectisome (34, 37, 38). The decrease in cytosolic levels of ExsE triggers partner switching wherein ExsC preferentially binds ExsD in a 2:2

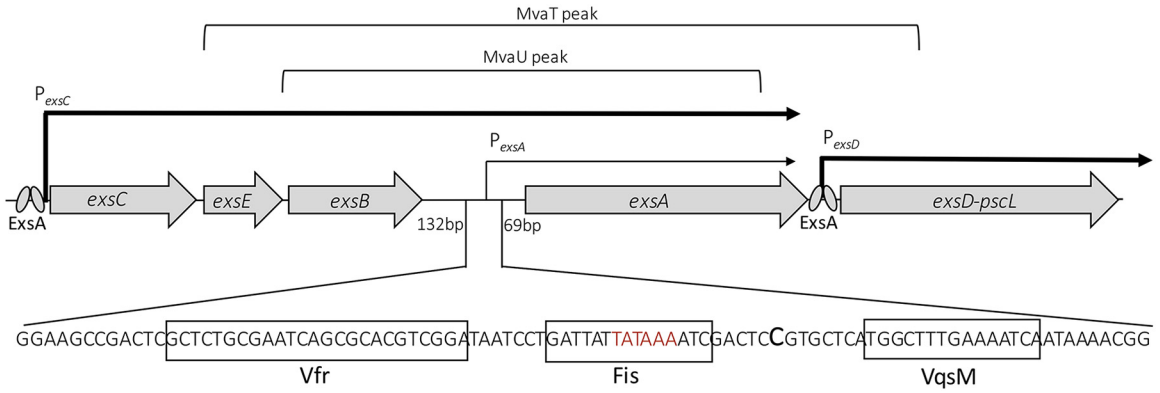

FIG 2 Map of the T3SS regulatory locus and locations of the P_{exsC} and P_{exsA} promoter regions. The binding sites for Vfr (30), Fis (58), and VqsM (57) are boxed. Brackets indicate the MvaT and MvaU binding regions based on ChIP-chip data (78). The P_{exsA} transcription start site is indicated with a large C, and the −10 region is in red.

complex, resulting in the release of ExsA (Fig. 1) (35, 36, 39–41). Liberated ExsA then binds to target promoters and recruits RNA polymerase to activate the T3SS regulon (28, 42, 43). Partner switching is likely driven by affinity differences in the protein-protein interactions (ExsE-ExsC > ExsD-ExsE > ExsA-ExsD, in decreasing order of affinity). While certainly true for the ExsE-ExsC (18 nM) and ExsD-ExsC (1 nM) interactions (35), the affinity of the ExsA-ExsD complex is unknown but predicted to be weaker than that for ExsD-ExsC. Partner-switching mechanisms similar to the *P. aeruginosa* system also regulate T3SS gene expression in *Photorhabdus luminescens* (44, 45), *Aeromonas hydrophila* (46–48), *Vibrio parahaemolyticus* (49–52), and *Vibrio alginolyticus* (53).

In addition to partner-switching-mediated control of ExsA activity, PtrA may also directly bind to and inhibit ExsA activity. PtrA is a copper-sensing protein upregulated in the presence of copper (54). Although one group found that PtrA interacts with ExsA to inhibit T3SS gene expression (54), a second group found that PtrA is a periplasmic protein important for copper tolerance and that PtrA does not interact with ExsA or play a role in the control of T3SS gene expression (55). To the best of our knowledge, no studies have observed direct repression of the T3SS by copper.

TRANSCIPTIONAL CONTROL OF ExsA

Transcription of *exsA* is driven from two distinct promoters. For many years, the P_{exsC} promoter, which produces the *exsCEBA* polycistronic mRNA, was the only known mechanism of generating *exsA* transcript (Fig. 2) (27). A recent genome-wide screen for transcription start sites identified a novel transcript originating 100 nucleotides upstream of the *exsA* start codon (56) (Fig. 2). The same start site was identified by 5′ rapid amplification of cDNA ends (RACE) within two nucleotides (30). The newly discovered promoter, designated $P_{exsA'}$ generates a monocistronic *exsA* transcript. The P_{exsC} promoter is ~400 times more active than P_{exsA} and thus makes a larger contribution to *exsA* transcript levels under inducing conditions. ExsA itself does not bind to or regulate P_{exsA} promoter activity (30). Nevertheless, the P_{exsA} promoter is subject to significant regulatory control, stimulated by Vfr, Fis, and VqsM and silenced by the histone-like proteins MvaT and MvaU (30, 57–59).

Control of P_{exsA} promoter activity. Vfr is a global regulator of *P. aeruginosa* virulence homologous to *Escherichia coli* cAMP receptor protein (CRP) (60). The cAMP-Vfr signaling system activates exotoxin A production (60, 61), type IV pilus biosynthesis (62), the *las* quorum sensing (QS) system (63), and T3SS gene expression (64) and inhibits flagellar gene expression (65). Coordinated regulation of the T3SS with type IV pili is noteworthy because the associated adherence function is necessary for intimate adherence to host cells and translocation of the T3SS effectors. Although the primary effect of calcium chelation is through activation of secretion, the activity of the

cAMP-Vfr signaling system is enhanced by EGTA (presumably through calcium chelation) (64). Because cAMP production is also stimulated by high osmolarity (64), cAMP-Vfr signaling contributes to T3SS gene control in response to hyperosmotic stress (34, 66). Vfr directly binds to and stimulates P_{exsA} promoter activity (30). The Vfr binding site is centered ~42 bp upstream of the P_{exsA} promoter transcription start site (Fig. 2). Curiously, disruption of the P_{exsA} Vfr binding site on the chromosome results in a more severe phenotype than does deletion of *vfr* itself (30). Whether the mutation disrupts other DNA-binding proteins that control P_{exsA} promoter activity, RNA-binding proteins that interact with the longer *exsCEBA* mRNA to regulate ExsA translation (see below), or *exsCEBA* mRNA stability is unclear.

Fis is a nucleoid-associated protein that binds to and bends DNA (67, 68). T3SS gene expression and T3SS-dependent cytotoxicity are reduced in a *fis* transposon insertion mutant (58). Although a Fis binding site is located between the Vfr binding site and the −10 region of the P_{exsA} promoter (Fig. 2), P_{exsA} promoter activity is unaffected in the *fis*::Tn mutant. Instead, the decrease in T3SS gene expression correlates with reduced P_{exsC} promoter activity. While the precise mechanism of control by Fis is unclear, there are a number of interesting connections between Fis and previously described regulators of the T3SS. Translation of Fis is inhibited by the small noncoding (sRNA) RgsA through direct base pairing with the *fis* mRNA (69). RpoS is essential for *rgsA* transcription, consistent with previous data showing that RpoS inhibits T3SS gene expression (70). Base pairing between RgsA and the *fis* mRNA is dependent upon the RNA chaperone Hfq and is consistent with recent data suggesting that Hfq inhibits T3SS gene expression (71). Finally, activation of the GacAS two-component system inhibits T3SS gene expression (72–74). While the primary effect of GacAS signaling is reduced cAMP-Vfr signaling and ExsA translation (75) (see below), *rgsA* transcription is indirectly stimulated by the GacAS two-component system (69).

VqsM is an AraC family transcriptional regulator which activates the *las* quorum sensing system and T3SS gene expression (57, 76). The reported VqsM binding site is located downstream of the transcription start site for the P_{exsA} promoter (30, 56, 57). Given the location of the binding site, it seems unlikely that VqsM activates the P_{exsA} promoter, raising the possibility of a second VqsM-dependent promoter that contributes to *exsA* transcription. The relative contribution of VqsM to T3SS gene expression appears to be strain specific, as *vqsM* is not found in all *P. aeruginosa* strains (77).

MvaT and MvaU are members of the histone-like nucleoid structuring (H-NS) family of global transcription repressors. H-NS proteins oligomerize on the DNA and silence gene expression by competing with transcription factors and/or trapping/occluding RNA polymerase (RNAP). A chromatin immunoprecipitation with microarray technology (ChIP-chip) study identified MvaT and MvaU binding sites in the P_{exsA} promoter region (Fig. 2) (78). Whereas strains lacking *mvaT* or *mvaU* demonstrate elevated P_{exsA} promoter activity and T3SS gene expression, overexpression of either MvaT or MvaU inhibits T3SS gene expression (59). Although an *mvaTU* double mutant is lethal, the depletion of *mvaU* by CRISPR interference in an *mvaT* mutant results in significant stimulation of P_{exsA} promoter activity, leading to the conclusion that MvaT and MvaU have redundant roles in silencing T3SS gene expression. The proposed model is that MvaT and MvaU bind the P_{exsA} promoter region and inhibit transcription until an activator overrides silencing (59). The best candidate is Vfr, as it appears to be required for P_{exsA} transcription regardless of whether *mvaT* and *mvaU* are present or absent (59). It remains possible, however, that Fis, VqsM, or an unknown factor also competes with MvaT/MvaU to override the silencing activity.

The ChIP-chip data did not show MvaT or MvaU binding to promoter regions of any other known regulator of *exsA* transcription, including *vfr* (59, 78). MvaT and MvaU also inhibit transcription of the small RNAs RsmY and RsmZ involved in sequestration of RsmA (59, 78, 79). Although the net effect might liberate RsmA, which has a positive effect on ExsA translation (discussed below) (74, 75, 78), regulation of *rsmYZ* transcription by MvaT/MvaU does not appear to have a significant effect on T3SS gene expression.

Control of P_{exsC} promoter activity. The P_{exsC} promoter is primarily activated by ExsA (27), resulting in a positive feedback loop with most *exsA*-containing transcript generated under inducing conditions originating from the P_{exsC} promoter (Fig. 2), which is ~400-fold more active than the P_{exsA} promoter (30). Full activation of P_{exsC} promoter activity is dependent upon PsrA, a TetR family member (80). PsrA is activated by long-chain fatty acids (LCFAs) such as oleate (81) and antimicrobial peptides (82). PsrA binds P_{exsC} with relatively low affinity compared to its own promoter (80). LCFAs activate *psrA* transcription by directly binding to PsrA and preventing self-repression (81). When LCFAs are at low levels, there is excess PsrA to activate P_{exsC}; however, when LCFA levels are high enough to bind all of the PsrA in the cell, PsrA-dependent transcription is inhibited (Fig. 3).

TRANSLATIONAL CONTROL OF ExsA

Following transcription, the *exsA* mRNA is subject to additional levels of regulatory control at the posttranscriptional level. Translation of *exsA* is positively regulated by the RNA-binding proteins RsmA and DeaD and negatively regulated by at least one small noncoding RNA (sRNA 0161), presumably with the assistance of Hfq (71, 72, 75, 83).

Translational control by RsmA. The Rsm system controls a critical lifestyle switch by inversely controlling phenotypes that favor acute infection or chronic colonization (84). The primary effector of the system is RsmA. Determinants positively controlled by RsmA include the cAMP-Vfr signaling system, type IV pili, and T3SS gene expression. Conversely, RsmA inhibits biofilm production, quorum sensing, the type VI secretion system (T6SS), and hydrogen cyanide (HCN) production (79, 85–88). An *rsmA* mutant has reduced colonization in an acute infection model, partially explained by a loss of T3SS gene expression and type IV pili, and enhanced persistence in a chronic infection model (89). This mirrors the general observation that *P. aeruginosa* isolates from chronic cystic fibrosis infections are defective for T3SS gene expression (90, 91). Although the significance of this observation is unclear, it suggests that T3SS gene expression is negatively selected against in the context of chronic colonization.

RsmA is an ortholog of *E. coli* CsrA, where it was identified as a carbon source regulator (85, 92). The CsrA family of proteins bind target mRNAs and negatively or positively modulate translation efficiency and/or stability (93). RsmA binds target mRNAs through the recognition of GGA motifs presented in the loop portion of stem-loop structures (94). For repressed targets, the RsmA binding site typically overlaps the ribosome binding site (RBS). RsmA stimulates T3SS gene expression through at least two mechanisms (Fig. 3). The first promotes cAMP-Vfr signaling and impacts T3SS gene expression through P_{exsA} promoter activity. The transcription of both *vfr* and *cyaB* is significantly reduced in the absence of *rsmA,* although the mechanism of control remains unknown (72). CyaB is the primary adenylate cyclase involved in the generation of cytoplasmic cAMP (64). The second stimulatory function of RsmA enhances ExsA translation ~2- to 3-fold (75). Although a 2- to 3-fold change in translation efficiency may appear trivial, small changes in any member of the partner-switching mechanism (ExsA, ExsD, ExsC, or ExsE) are likely sufficient to trigger significant changes in T3SS gene expression (95). It is not known whether RsmA functions directly or indirectly to stimulate Vfr and ExsA translation. Another potential explanation for a loss of T3SS gene expression in an *rsmA* mutant is elevated levels of c-di-GMP, which appears to antagonize cAMP-Vfr signaling (described below) (96, 97). The combined effects of RsmA on *exsA* transcription and translation are substantial, as *rsmA* and *exsA* mutants are equally defective for T3SS gene expression (75).

RsmA activity is controlled by several "decoy" sRNAs, including RsmV, RsmW, RsmY, and RsmZ, with RsmY and RsmZ playing the most prominent roles. Each sRNA has multiple RsmA binding sites (86, 94, 98–102). High levels of the sRNAs result in the sequestration of RsmA from target mRNAs and reduced T3SS gene expression. Cellular levels of RsmY and RsmZ are controlled by the GacAS two-component regulatory system (Fig. 3). GacS is an unusual histidine kinase with H1/D1/H2 (autophosphorylating histidine, receiver aspartate, and second histidine, respectively) domains (103).

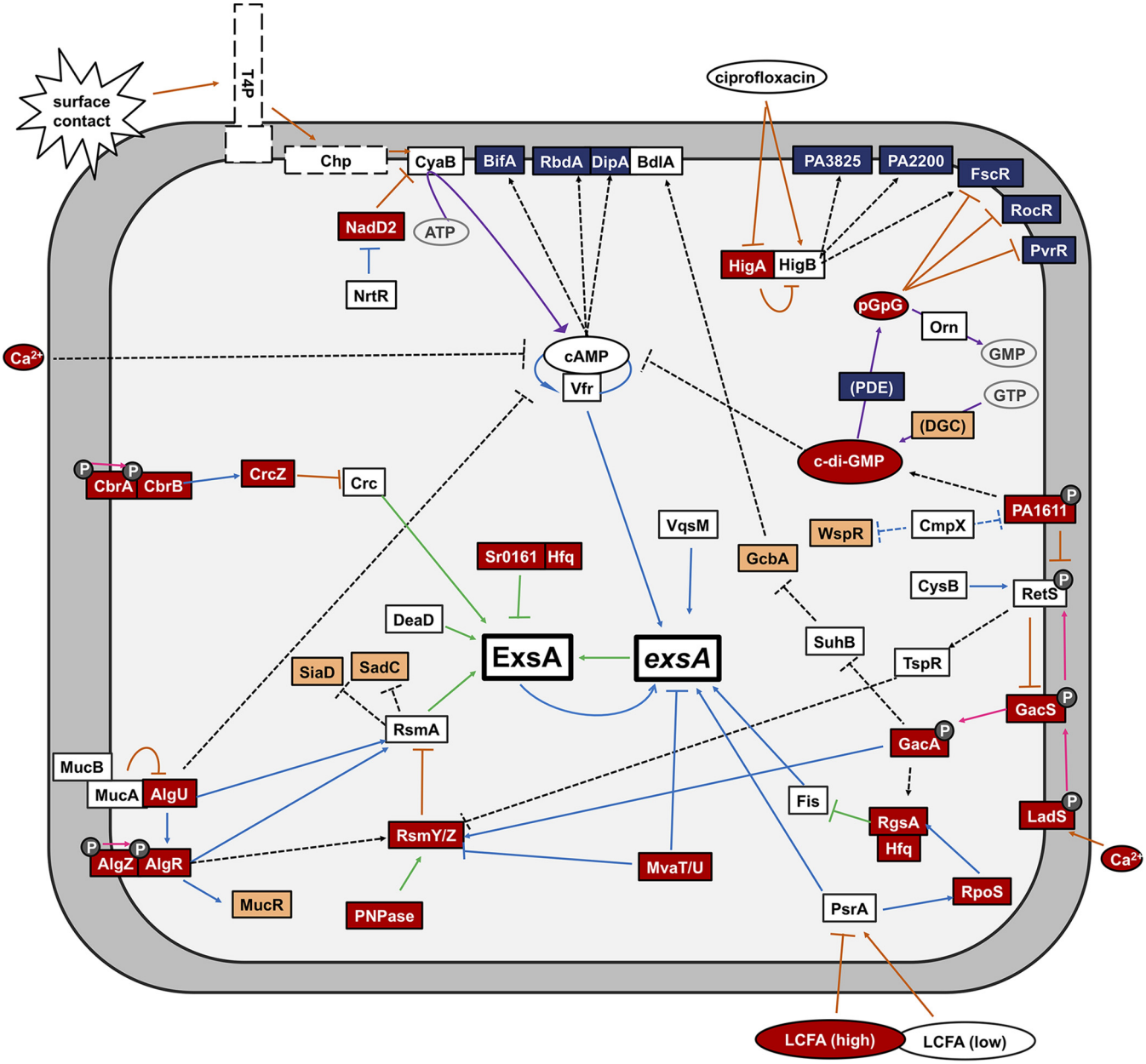

FIG 3 Regulatory pathways that control T3SS gene expression. Gene products that stimulate T3SS gene expression are shown in white, while those that inhibit are red. Diguanylate cyclases (DGC) that synthesize c-di-GMP are orange, and phosphodiesterases (PDE) that degrade c-di-GMP are blue. P indicates a histidine kinase or response regulator. Blue lines signify transcriptional regulation, green lines signify posttranscriptional regulation, orange lines signify control of protein activity, purple lines signify enzymatic activity, pink lines signify phosphotransfer activity, and black dashed lines are links with indirect or unknown mechanisms.

GacA is a response regulator (RR) whose sole function is activation of *rsmY* and *rsmZ* transcription following phosphorylation by GacS (74). Phosphotransfer from GacS to GacA is regulated by several additional histidine kinases. The RetS hybrid histidine kinase positively regulates the T3SS by dimerizing with the GacS H1 domain and blocking GacS autophosphorylation and by dephosphorylating GacS, preventing phosphotransfer to GacA (104–106). PA1611, another hybrid histidine kinase, counteracts RetS-mediated interference by forming heterodimers with RetS (107). The LadS histidine kinase activates GacS by transphosphorylating the GacS H2 domain (103). LadS is activated by extracellular calcium leading to the induction of *rsmYZ* transcription and sequestration of RsmA. Reduced RsmA availability results in an inhibition of T3SS gene expression and expression of genes associated with chronic colonization (108).

Several additional factors that control T3SS gene expression are linked to the Rsm system. Polynucleotide phosphorylase (PNPase) inhibits T3SS gene expression by directly stabilizing RsmY and RsmZ, leading to enhanced RsmA sequestration (109). TspR, a protein of unknown function, is encoded immediately downstream of *retS* and indirectly activated by RetS (110). T3SS gene expression and ExsA translation are reduced in a *tspR* mutant and restored in a *tspR* mutant lacking *rsmY* and *rsmZ* (110). TspR appears to stimulate T3SS gene expression by suppressing *rsmY* and *rsmZ* transcription, potentially via effects on RetS. CysB is a LysR family transcription factor that contributes to T3SS gene expression by controlling *retS* transcription (111). Although *E. coli* CysB activates sulfate assimilation, *retS* transcription is unaffected by sulfate or cysteine addition (111).

Translational control by the RNA helicase DeaD. DEAD proteins are ubiquitous ATP-dependent RNA helicases with a conserved Asp-Glu-Ala-Asp (DEAD) motif. Roles of DEAD box proteins include ribosome biogenesis, translation initiation, RNA decay, and growth promotion at low temperature (112). *P. aeruginosa deaD* was identified by screening transposon libraries for defects in T3SS activity (113, 114). The lack of T3SS gene expression in a *deaD* mutant results from reduced *exsA* translation (2- to 3-fold) without impacting mRNA stability (114). Although the *P. aeruginosa* genome contains 7 DEAD box helicases, only *deaD* is essential for T3SS gene expression (114). The native *exsA* RBS is required for DeaD-mediated activation, and Mfold predictions suggest that the mRNA adopts a confirmation that partially occludes the RBS (114). Purified DeaD is sufficient to stimulate *exsA* translation *in vitro* leading to a model wherein DeaD functions by altering the mRNA structure and enhancing ribosomal access. The RsmA and DeaD requirements for *exsA* translation have not been fully defined, but both appear to be essential, as *rsmA* or *deaD* provided in *trans* is unable to complement the *deaD* or *rsmA* mutants, respectively (114).

Translational control by sRNA 0161 and Hfq. Whereas RsmY and RsmZ are sRNAs that modulate protein activity (i.e., RsmA and RsmF), other sRNAs regulate gene expression by imperfectly base pairing with the target mRNAs (115). These sRNAs (typically 50 to 300 nucleotides [nt]) often base pair with the mRNA at or near the RBS and block translation by preventing ribosome binding. In Gram-negative bacteria, the RNA chaperone Hfq is usually required for sRNA function and/or stability. A high-throughput global sRNA target identification by ligation and sequencing (Hi-GRIL-seq) screen identified sRNAs and their cognate target mRNAs in *P. aeruginosa* (71). sRNA Sr0161 was found to inhibit *exsA* translation, and overexpression of Sr0161 reduced T3SS gene expression (71). Other sRNAs target Fis and RpoS, both of which contribute to T3SS gene expression (69, 116).

Translational control by Crc. The Crc protein controls the availability of enzymes and transporters involved in the utilization of secondary carbon sources. A *crc* mutant has reduced T3SS expression, swimming, swarming, twitching, and initial biofilm formation. A microarray study identified 428 differentially controlled genes in a *crc* mutant, including several with described roles in T3SS gene control (117). The primary explanation for reduced T3SS gene expression is that Crc appears to stimulate ExsA translation (117). Although Crc and Hfq can function together to repress the translation of target genes (118–123), *hfq* and *crc* mutants have opposite phenotypes for T3SS gene expression (117, 122). It is unlikely, therefore, that Hfq and Crc work together to directly control the T3SS. Like RsmA, Crc activity is controlled by an sRNA, CrcZ, which acts by sequestering Crc from target mRNAs (124). The expression of *crcZ* is controlled by the CbrAB two-component system in response to different carbon sources (124). This has potential implications to previous studies showing that metabolic activity controls T3SS gene expression (34).

SECOND MESSENGERS

Second messengers are intracellular signaling molecules produced in response to a "first messenger," usually an extracellular signaling molecule. The second messengers cAMP and bis-(3′-5′)-cyclic dimeric GMP (c-di-GMP) serve as regulatory switches that

alter cell growth, motility, lifestyle, and virulence (125, 126). Whereas cAMP directly binds to and activates Vfr to promote T3SS gene expression (60), c-di-GMP inhibits the T3SS.

cAMP. As discussed above, cAMP allosterically activates the DNA-binding activity of Vfr, leading to enhanced transcription of target promoters, including P_{exsA} (30, 64, 127). cAMP is synthesized by two adenylate cyclases, CyaA and CyaB (64, 128). CyaA is located in the cytoplasm and plays a minor role in cAMP synthesis. Most cAMP is synthesized by the inner membrane-anchored CyaB (64, 129). cAMP homeostasis is maintained by CpdA, a phosphodiesterase that degrades cAMP to 5′-AMP (128). cAMP production is regulated by type IV pili and the Chp chemotaxis-like system (130, 131). Mechanosensation of surfaces by type IV pili is thought to activate the Chp system, stimulate CyaB activity, and induce expression of Vfr-dependent surface-associated virulence phenotypes (Fig. 3) (132–134).

NrtR is a transcription factor that represses itself and *nadD2*, a nicotinate mononucleotide adenylyltransferase gene. NrtR is required for T3SS gene expression, infection of HeLa cells, and colonization in an acute murine pneumonia model (113, 135). Loss of T3SS gene expression in the *nrtR* mutant results from a reduction in cAMP levels. The current model proposes that NadD2 directly interacts with and inhibits CyaB activity (135).

c-di-GMP. c-di-GMP regulates polysaccharide synthesis, biofilm formation, adherence, and virulence factors and controls the transition between planktonic and sessile lifestyles (acute to chronic infection) (136–138). Expression of the T3SS is inhibited by elevated levels of c-di-GMP (125, 139–141). c-di-GMP is synthesized from two molecules of GTP by diguanylate cyclases (DGC) and degraded to either pGpG or two GMPs by phosphodiesterases (PDEs). pGpG is hydrolyzed into two GMP molecules by oligoribonuclease (Orn) (142, 143). Because pGpG inhibits the activity of some PDEs (e.g., RocR) (142, 143), a loss of Orn leads to an increase in cellular c-di-GMP and inhibition of the T3SS (144).

Overexpression of some DGCs inhibits cAMP-Vfr-dependent reporter activity, and overexpression of some PDEs stimulates reporter activity. DGCs with potential relevance to T3SS gene control include WspR, SiaD, SadC, MucR, and GcbA, and implicated PDEs include PvrR, RocR, FcsR, PA2200, PA3825, NbdA, BifA, DipA, and RbdA (96, 113, 126, 141–143, 145–152) (Fig. 3). The relative contribution of each has been challenging to sort out. The observation that c-di-GMP and cAMP levels are inversely related to one another likely accounts for the effect of c-di-GMP on T3SS gene expression (125, 126). The mechanistic basis for this reciprocal relationship is unclear but does not involve c-di-GMP-mediated changes to *cyaAB* or *vfr* transcription or translation or alteration of CyaA, CyaB, or CdpA activity (125). Involvement of the Chp system in cAMP production may provide a link, as both ChpA and PilK are putative c-di-GMP binding proteins (153).

SuhB is an inositol monophosphatase essential for virulence in a murine model (113, 154). SuhB inhibits the DGC GcbA, resulting in decreased c-di-GMP levels (Fig. 3) (146). SuhB also inhibits the expression of GacA (113). Because RsmY and RsmZ levels are increased in a *suhB* mutant, ExsA translation is dependent on the presence of *suhB*.

The HigB-HigA toxin/antitoxin system stimulates T3SS gene expression when cells are exposed to ciprofloxacin (Fig. 3) (148, 155). Deletion of the HigA antitoxin results in higher levels of free HigB, reduced c-di-GMP, and increased T3SS gene expression (155). Free HigB results in increased levels of three PDEs (FcsR, PA2200, and PA3825). The deletion of all three PDEs results in elevated c-di-GMP and inhibition of the T3SS phenotype (148). This is contrary to the findings from another study, however, which showed that ciprofloxacin treatment inhibits the T3SS (156).

Activation of MucA/AlgU signaling inhibits T3SS gene expression. Chronic colonization of the cystic fibrosis airways is associated with mucoid conversion and loss of T3SS gene expression (90, 91, 157, 158). The mucoid phenotype results from alginate overproduction and is controlled by the MucA/AlgU regulatory cascade. MucA is an anti-sigma factor that sequesters the AlgU sigma factor (159). Inactivating mutations in

mucA are common in chronically adapted *P. aeruginosa* isolated from cystic fibrosis lungs, and those mutations result in activation of the AlgU regulon (158). The AlgU regulon includes genes required for alginate biosynthesis and *algR*, which encodes a response regulator that controls alginate production, type IV pili, and virulence (158, 160). AlgR is part of a two-component system with AlgZ serving as the histidine kinase (161). cAMP-Vfr-dependent signaling and the Rsm system are both altered in *mucA* mutants (75, 162–164). AlgR and AlgU directly activate *rsmA* expression (165, 166), and AlgR increases RsmY and RsmZ expression through an undefined mechanism (75). The net effect of stimulating *rsmA*, *rsmY*, and *rsmZ* transcription is enhanced sequestration of RsmA (75). The lack of T3SS gene expression in a *mucA* mutant, therefore, results from reduced RsmA availability, which decreases Vfr-dependent P_{exsA} transcription and RsmA-stimulated translation of ExsA, as described above (Fig. 3). The T3SS defect in strains lacking *mucA* can be largely suppressed by increasing cAMP-Vfr signaling and the levels of free RsmA (75). AlgR also directly activates *mucR*, a DGC, but the relative contribution of this to T3SS gene control has not been examined (152). MucB, also required for T3SS gene expression, is a periplasmic protein that binds to and protects MucA from proteolytic cleavage by AlgW (113, 167, 168). Although mucoid conversion is associated with the onset of irreversible colonization of the cystic fibrosis airways, the finding that the losses of cAMP-Vfr signaling and T3SS gene expression are coincident events suggests that the selective advantage conferred to *mucA* mutants in the cystic fibrosis airways may extend beyond mucoidy.

MISSING LINKS

Several genes/pathways that affect T3SS gene expression lack obvious connections to known mechanisms of T3SS gene control. The realization that most upstream regulators impact *exsA* transcription and/or ExsA synthesis through cAMP-Vfr, the RsmA system, and/or c-di-GMP provides a framework to rapidly evaluate regulators of unknown function.

PtrB, which is activated by degradation of the PtrR repressor following DNA damage, is a repressor of T3SS gene expression (169). Overexpression of efflux pumps MexCD-OprJ and MexEF-OprN, as well as PtrC, also inhibits T3SS gene expression (170, 171). The spermine/spermidine uptake transporter is required for T3SS gene expression (172). Spermine and spermidine are polyamines that can be utilized by *P. aeruginosa* as sole carbon and nitrogen sources (173, 174). While spermidine can be synthesized by *P. aeruginosa*, spermine is only found in eukaryotes. Mutants in the spermidine uptake transporter, but not synthesis genes, are deficient for T3SS gene expression, indicating that spermine and spermidine could be host signals that activate the T3SS (172). This is consistent with the finding that the addition of exogenous spermidine or spermine increases T3SS gene expression and cytotoxicity (172). The cAMP-Vfr and Rsm systems are unaffected in cells lacking the spermine transporter, suggesting that T3SS activation occurs through a novel mechanism.

The heat shock protein DnaK is required for acute virulence and T3SS gene expression (113, 154, 175). Elastase activity, exotoxin A secretion, swimming, swarming, and twitching motility are also diminished in a *dnaK* mutant, leading to the possibility that the phenotypes reflect a defect in cAMP-Vfr signaling (175). DnaK colocalizes in the periplasm with the nitrite reductase NirS (176). NirS inhibits c-di-GMP production and is required for T3SS gene expression (177).

THERAPEUTICS AND INHIBITORS OF T3SS GENE EXPRESSION

The essential role of the T3SS in the context of acute infection makes it an attractive target for therapeutic development. Targetable events include T3SS gene expression, injectisome assembly, secretory activity, effector translocation, and effector activity. Because T3SS gene expression is coupled with secretory activity by partner switching, drugs that inhibit assembly or secretion should also significantly reduce T3SS gene expression.

N-Hydroxybenzimidazoles were originally identified using a structure-based ap-

proach as inhibitors of AraC proteins (178). Several *N*-hydroxybenzimidazoles with 50% inhibitory concentrations (IC_{50}s) in the low-micromolar range inhibit the DNA-binding activity of ExsA *in vitro* and T3SS-dependent toxicity toward macrophages (179). The *N*-hydroxybenzimidazoles interact with the carboxy-terminal DNA-binding domain of ExsA to inhibit DNA-binding activity (180). *N*-Hydroxybenzimidazoles also inhibit the DNA-binding activity of ExsA orthologs from *Yersinia pestis*, *Aeromonas hydrophila*, *Photorhabdus luminescens*, and *Vibrio parahaemolyticus* (180). Although active against ExsA *in vitro*, *N*-hydroxybenzimidazoles do not inhibit ExsA-dependent promoter activity *in vivo* when using either *P. aeruginosa* or *E. coli* reporter strains. The significant reduction in T3SS-dependent toxicity observed in coculture experiments with Chinese hamster ovary cells, therefore, is enigmatic. It is interesting to note that *N*-hydroxybenzimidazoles display broad-spectrum activity against distantly related AraC proteins (178, 181) and that the *P. aeruginosa* genome has >50 AraC family members. The protective activity of *N*-hydroxybenzimidazoles may result from effects on multiple AraC proteins or off-target effects.

As discussed above, spermidine transport is necessary for T3SS gene expression (172). Evidence for the efficacy of targeting spermidine was shown using a rhodamine 101-spermine conjugate that inhibits spermidine uptake, T3SS gene expression, cytotoxicity toward HeLa cells, and virulence in mice (182). This was followed by generating a spermidine IgG1 monoclonal antibody. The antibody also prevents spermidine-dependent activation of the T3SS, protects A549 cells for toxicity, lowers spermidine levels in mouse serum, and protects mice from *P. aeruginosa* lung infection (183).

Although the macrolide azithromycin does not kill planktonic *P. aeruginosa* at typically achieved clinical concentrations, sublethal doses inhibit quorum sensing and biofilm formation and increase T3SS gene expression (184–188). The biofilm and T3SS phenotypes likely result from inhibition of *rsmY* and *rsmZ* expression by azithromycin, leading to an increase in RsmA availability (189). Elevated T3SS gene expression is consistent with the findings that azithromycin treatment results in greater cytotoxicity when *P. aeruginosa* is cocultured with J774.A1 cells and increased mortality in mice following pretreatment with macrolides (190, 191). Azithromycin has positive therapeutic benefits for patients with chronic *P. aeruginosa* pulmonary disease, possibly by reducing biofilm formation (192–194). The efficacy of azithromycin in chronic infection may also reflect the tendency of chronic *P. aeruginosa* isolates to be defective in T3SS function (90, 91).

Several plant phenolic compounds (salicylic acid, its precursors, and their analogues) inhibit or activate T3SS gene expression (195). The compound TS027 inhibits *exsA* transcription likely through effects on ExsA translation via the Rsm system (195). Compound TS103 activates the T3SS, probably by inhibiting *rsmYZ* transcription and increasing RsmA availability. Coumarin is a phenolic compound that inhibits T3SS gene expression during planktonic growth by an unknown mechanism (196).

Other inhibitors of the T3SS include the anticancer drug cisplatin (197), salicyclidene acylhydrazide INP0341 (198), (−)-hopeaphenol (199), a selection of synthetic cyclic peptomers (200), and the small-molecule inhibitor fluorothiazinon (197, 201). Many of these compounds protect eukaryotic cells from T3SS-induced cytotoxicity (198, 199, 201).

CONCLUSIONS

The T3SS represents a mechanism to protect *P. aeruginosa* from predators ranging from amoebae to human neutrophils. Activation by the partner-switching cascade appears to be simplistically brilliant. Host cell contact is a highly relevant signal and triggers immediate induction of T3SS gene expression. It is puzzling, therefore, that so many additional resources are dedicated to controlling the T3SS. One potential explanation is that the signaling systems positioned above the partner-switching mechanism fine-tune T3SS gene expression. Why fine-tuning would be required is not obvious, as the requirement to express the T3SS gene expression seems straightforward: activate when a threat is present and inhibit when the threat has passed. Another explanation

may relate to the bistability of T3SS gene expression. Single-cell experiments with fluorescent reporters have revealed that only a subset of *P. aeruginosa* cells within a population induce T3SS gene expression in response to either low calcium or cultured mammalian cells (34, 95, 202, 203). This phenomenon, referred to as bistability, results from stochastic fluctuations in the production and/or activity of a transcription factor (204). Bistable expression of the T3SS likely results from the combined effects of ExsA autoregulating its own transcription, ExsA-dependent control of the ExsD anti-activator, and the partner-switching mechanism. Bistable expression of the T3SS may promote social cheating. Social cheating occurs when certain members of a population produce a beneficial trait, such as T3SS-mediated killing of immune cells, and other members (cheaters) take advantage without incurring the costs associated with producing the benefit (205). A recent study found that ~80% of *P. aeruginosa* cells express the T3SS in an acute murine infection model (206). In competition experiments, wild-type (wt) *P. aeruginosa* has a competitive advantage over a strain overexpressing the T3SS and a disadvantage against T3SS-negative strains (206). The fitness cost associated with T3SS gene expression may be energetic and/or related to expression of immunogenic molecules, such as the needle complex tip protein PcrV (207). When T3SS-defective strains are provided in significant excess over wt organisms, the disadvantage to wt organisms is negated (206). These findings may explain why T3SS-negative bacteria can be isolated from acute infections (208, 209). Cheater populations usually become unstable when the proportion of producers to cheaters becomes excessive. Fine-tuning T3SS gene expression in response to a wide range of environmental signals may establish an optimal bistable population and afford the greatest fitness advantage for cells that both express and repress T3SS gene expression.

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