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MINIREVIEW

Nooks and Crannies in Type VI Secretion Regulation[▽]

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Type VI secretion systems (T6SS) are macromolecular, transenvelope machines encoded within the genomes of most Gram-negative bacteria, including plant, animal, and human pathogens, as well as soil and environmental isolates. T6SS are involved in a broad variety of functions: from pathogenesis to biofilm formation and stress sensing. This large array of functions is reflected by a vast diversity of regulatory mechanisms: repression by histone-like proteins and regulation by quorum sensing, transcriptional factors, two-component systems, alternative sigma factors, or small regulatory RNAs. Finally, T6SS may be produced in an inactive state and are turned on through the action of a posttranslational cascade involving phosphorylation and subunit recruitment. The current data reviewed here highlight how T6SS have been integrated into existing regulatory networks and how the expression of the T6SS loci is precisely modulated to adapt T6SS production to the specific needs of individual bacteria.

Bacterial pathogenesis is controlled by the coordinated action of a number of virulence factors, including those involved in adhesion, biofilm formation, invasion, and secretion of protein toxins. Most of these attributes depend on specific and dedicated organelles such as flagella, pili, adhesins, exopolysaccharides, and protein secretion systems, which need to be tightly regulated to make the pathogenesis a highly efficient and powerful process. Recently, a new actor in bacterial pathogenesis has been identified, the type VI secretion systems (T6SS) (75, 87). T6SS are encoded within the genomes of most Gram-negative bacteria, including plant, animal, and human pathogens, as well as environmental strains (10, 13, 22, 41). Type VI secretion genes encode cytoplasmic, periplasmic, and membrane proteins that assemble to form a transenvelope apparatus. Interestingly, two subunits regularly found in culture supernatants of strains carrying T6SS, Hcp and VgrG, are structural homologues of the bacteriophage T4 gp19 and gp27-gp5 proteins, which constitute the tail and the syringe of the baseplate, respectively (57, 63, 75, 83, 86). Based on these homologies, it has been proposed that the assembled secretion system may resemble an upside-down bacteriophage (56, 63) in which the additional bacteriophage-unrelated genes may participate in the assembly of the structure or its stabilization. Indeed, two subunits resemble the IcmF and IcmH proteins that stabilize the type IV secretion systems in *Legionella pneumophila* (22, 33), whereas other T6SS-associated genes encode putative cytoplasmic proteins, an outer membrane lipoprotein (3), or inner membrane proteins, including a cell wall binding protein that anchors the secretion system to the peptidoglycan layer (4). The virulence factors secreted through these apparatuses are not identified yet, but in several cases, the VgrG proteins carry a supplementary C-terminal effector module (86, 88). These extensions have homologies to proteins involved (i) in the remodeling of the cytoskeleton through processes such as actin cross-linking and ADP-ribosylation (68, 86, 88, 100), (ii) in motility, such as tropomyosin, (iii) in apoptosis, such as cathepsin D, (iv) in adhesion, such as YadA-like adhesin and mannose binding domains, or (v) in binding to the extracellular matrix, such as peptidoglycan and fibronectin (86, 88). However, in many cases, the VgrG proteins do not carry an extension, and these secretion systems are probably not involved in the delivery of effector modules but may have evolved for other functions such as biofilm formation, sensing of the environment, or response to stress. For example, a recent study reported that the *Pseudomonas aeruginosa* Hcp secretion island 1 (HSI-1) type VI secretion gene cluster is required for the secretion of a bacterial toxin that kills neighboring bacteria (50). Similar to colicin-producing bacteria (23), the producing bacterium is protected through the synthesis of an antitoxin protein (50). We refer the reader interested in general notions on T6SS or on the structure and function of the VgrG proteins to recent reviews (10, 13, 22, 41, 88).

Several phenotypes have been correlated with T6SS function: virulence of *Burkholderia cenocepacia* toward mice, survival of *Burkholderia mallei* and *Aeromonas hydrophila* in macrophages, resistance of *Vibrio cholerae* to amoeba predation, *Rhizobium leguminosarum* symbiosis, biofilm formation by enteroaggregative *Escherichia coli*, intracellular growth of *Francisella tularensis* and *Salmonella enterica* in macrophages, and stress sensing in *Vibrio anguillarum* (3, 11, 53, 62, 82, 87, 94, 95, 101, 111). In most cases for Gram-negative pathogens, T6SS are not critical determinants of pathogenesis but rather improve the efficiency of several stages during colonization and/or infection. T6SS are therefore found in a wide variety of Gram-negative bacteria, from environmental strains to pathogens, and are involved in a vast variety of processes. Although the contribution of T6SS to virulence has not been demon-

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strated or characterized yet for most T6SS-producing microorganisms, it appears that T6SS are adapted to the specific needs of each individual bacterium. Accordingly, the gene clusters encoding these machines should be precisely regulated by host functions and/or environmental conditions. Indeed, several studies have reported the identification of regulatory determinants and mechanisms underlying T6SS gene cluster expression. This expression involves a variety of transcriptional regulatory networks—including two-component systems (TCS), quorum-sensing (QS) systems, alternative sigma factors, and histone-like proteins—that are tightly linked to the expression of other virulence determinants. Interestingly, most T6SS gene clusters are found within pathogenicity islands probably acquired by horizontal transfer (10, 22), which implies that these genes have been integrated into preexisting regulatory networks. The currently available data are recapitulated in Table 1. In several species, T6SS remain inactive upon production. Their activation lies in a posttranslational regulatory cascade which requires a serine-threonine kinase and a phosphatase that regulate the phosphorylation status of a forkhead-associated (FHA) inner membrane protein and the recruitment of essential subunits to the apparatus (76). Understanding these regulatory networks and the corresponding responses is crucial to our overall knowledge of T6SS. In this review, we summarize the current knowledge regarding the regulatory proteins and mechanisms that modulate the expression of type VI secretion genes and activities and the interconnections with other bacterial virulence determinants.

TYPE VI SECRETION GENE CLUSTERS AND THE HOST

T6SS, like other virulence or adaptation factors, need to be tightly regulated. For this, bacteria are capable of sensing modifications in their environment and generating appropriate responses. In general, signal transduction systems constitute the basis of this sensing mechanism, and characterizing both the nature of the signals and the regulatory cascades is critical to understanding how T6SS contribute to adaptation, adhesion, or pathogenesis. Although the signals triggering T6SS gene expression are unknown, several environmental conditions have been reported to influence the production of type VI secretion machines (Table 1).

Expression of T6SS genes inside macrophages has been demonstrated for several pathogens. The *Burkholderia pseudomallei* Tss5 and *S. enterica* Sci T6SS and the *Francisella* pathogenicity island (FPI) T6SS of *F. tularensis* are upregulated inside macrophages (30, 34, 62, 82, 97); indeed, the *sci* cluster is necessary for delaying phagocytosis, whereas the FPI cluster is necessary for intracellular survival. Similarly, the *V. cholerae* T6SS *vas* genes are expressed in a rabbit ileal loop model of infection (33). Upon phagocytosis, production of the Vas T6SS induces cytoskeleton rearrangements through the secretion of the actin cross-linking domain of VgrG (68). The presence of circulating antibodies against the *P. aeruginosa* Hcp protein in the sera of cystic fibrosis patients (75) and the elevated levels of the HSI-1 Hcp protein produced by strains isolated from these patients (8, 75) suggest that the HSI-1 locus is induced in humans. Although no data for the *P. aeruginosa* HSI-2 locus within the host are available, the HSI-3 gene cluster is upregulated in the presence of epithelial cell extracts (32). In *Yersinia pestis*, the YPO0499 gene cluster is induced at low temperatures and repressed at 37°C, suggesting that the role of the T6SS gene cluster is probably more important for dissemination in the flea vector than in human cells (29, 31, 47, 49, 73, 85, 93).

A critical role for temperature-dependent induction of a T6SS gene cluster has also been observed for the *R. leguminosarum imp* locus (11). In *Pectobacterium atrosepticum*, the T6SS gene cluster is induced by the presence of potato tuber extracts (69, 70), whereas the *Agrobacterium tumefaciens* T6SS is induced under acidic conditions in the presence of low concentrations of phosphate, conditions found in proximity to wounded plant cells (115). Similarly, the T6SS gene cluster of the plant pathogen *Pseudomonas fluorescens* is induced by the presence of necrotic roots (7). The expression of several T6SS, including those of *Vibrio parahaemolyticus*, *P. aeruginosa* HSI-1 and HSI-3, and *Marinobacter hydrocarbonoclasticus*, is upregulated in static cultures or in biofilm compared to expression under planktonic conditions (1, 2, 38, 99, 105, 108). Interestingly, the *P. aeruginosa* HSI-1 and HSI-3 loci are induced at different times during biofilm development: the expression of HSI-1 increases progressively during biofilm formation (from attachment to maturation), whereas HSI-3 is temporarily induced during the initial steps of biofilm development (99). Interestingly, the RetS/Gac/Rsm pathway (a regulatory cascade that controls biofilm formation [45, 106]) is involved in the regulation of the *P. aeruginosa* HSI-1 gene cluster (14, 15, 75) (see below).

The *Edwardsiella tarda evp* cluster is induced at low temperatures (89) or in the presence of iron or copper (52, 109). Regulation by iron levels has been reported for *F. tularensis* and *Y. pestis* (35, 64, 84) and for the enteroaggregative *E. coli sci-1* gene cluster (Y. R. Brunet and E. Cascales, unpublished results).

TRANSCRIPTIONAL REGULATION OF TYPE VI SECRETION GENE CLUSTERS

Histone-like proteins: H-NS or H-NS-like proteins. Regulation of type VI secretion gene clusters by members of the H-NS family has been reported. These histone-like proteins function as global regulators by controlling the expression of a large number of genes throughout the genome (39, 78). H-NS binds to AT-rich regions of the chromosome and represses the transcription of the gene in the vicinity. As a consequence, many genes and clusters that have been horizontally acquired are silenced by H-NS (6, 46, 79). This is the case for several T6SS gene clusters, like that in *S. enterica* (67). In *P. aeruginosa*, the nucleoid-associated H-NS-like protein MvaT represses the HSI-2 and HSI-3 T6SS gene clusters (24) (Fig. 1). A recent study reported silencing of the *Pseudomonas putida* T6SS gene cluster by the TurA H-NS protein (92). Because most T6SS gene clusters have been acquired through horizontal transfer, this list is probably incomplete, and further studies will likely demonstrate the important role of histone-like proteins in the regulation of these clusters. Roles for other general nucleoid-associated proteins, such as the integration host factor (IHF), have been suggested in several cases (notably in the case of σ^{54}-regulated clusters [54]; see below), but an experimental demonstration is currently missing.

TABLE 1. Regulation of type VI secretion gene clusters

Organism	Gene cluster	Regulation profile or environmental and/or protein regulator(s)[a]	Analytical technique[b]	Reference(s)
Aeromonas hydrophila		AhyRI (QS system)	Western blotting	59
		VasH (σ^{54}-dependent activator)	Western blotting	101
Agrobacterium tumefaciens		Induction by acidic conditions	DNA microarray analysis	115
Burkholderia cenocepacia		CepIR (QS system)	*In silico* analysis	26
		CepIR and CciIR (QS systems)	DNA microarray analysis	80
		CepIR and CciIR (QS systems)	RT-PCR	80
		CepIR and CciIR (QS systems)	Reporter fusion	80
		AtsR (RetS-like protein)	Western blotting	5
Burkholderia mallei	*tss1*	VirAG (TCS)	DNA microarray analysis	95
		BMAA1517 (AraC-like TF [homologue of *B. pseudomallei* BrpC])	DNA microarray analysis	95
Burkholderia pseudomallei	*tss5*	Induction in macrophages	IVET	97
		Induction in macrophages	Reporter fusion	97
		BrpC (AraC-like TF)	RT-PCR	102
		BspR (TetR), BprP (OmpR), BsaN (AraC)	RT-PCR	102
Edwardsiella tarda	*evp*	Induction by copper	DNA microarray analysis	52
		Induction at low temp	Proteome analysis	89
		Induction by EsrAB (TCS)	Proteome analysis	89
		EsrC (AraC-like TF)	Proteome analysis	116
		EsrC (AraC-like TF)	Reporter fusion	116
	evpP	EsrAB (TCS)	RT-PCR	109
		Iron limitation	RT-PCR	109
		Fur	FURTA	109
Enteroaggregative *E. coli*	*sci-2*	AggR (AraC-like TF)	DNA microarray analysis	37
		AggR (AraC-like TF)	Reporter fusion	37
Francisella tularensis		Stringent response alarmone ppGpp	Reporter fusion	27
		MglA	Proteome analysis	62
		MglA	RT-PCR	62
		MglA	Reporter fusion	27, 28
		MglA	DNA microarray analysis	28
		MglA	DNA microarray analysis	17
		MglB	Western blotting	34
		SspA	DNA microarray analysis	28
		SspA	Reporter fusion	27
		Stringent response alarmone ppGpp	Reporter fusion	27
		PigR	Reporter fusion	27
		FevR (MerR-like TF)	DNA microarray analysis	16
		PmrA (RR/TCS)	DNA microarray analysis	72
		PmrA (RR/TCS)	EMSA	9
		PmrA (RR/TCS)	RT-PCR	9
		KdpD (sensor/TCS)	DNA microarray analysis	9
		MigR	RT-PCR	18
		Induction in mouse macrophages (intracellular)	Western blotting	34
		Induction by iron limitation	Proteome analysis	64
		Induction by iron limitation	Proteome analysis	35
		Induction by iron limitation	RT-PCR	35
		Induction by iron limitation	DNA microarray analysis	35
		Induction by iron limitation	Reporter fusion	18
		Fur	*In silico* analysis	35
Marinobacter hydrocarbonoclasticus		Induction in biofilms	Proteome analysis	105
Pectobacterium atrosepticum		Induction by potato tuber extracts	Proteome analysis	69
		Induction by potato tuber extracts	Northern blotting	69
		Induction by potato tuber extracts	RT-PCR	69
		Induction by potato tuber extracts	DNA microarray analysis	70
		ExpI (QS system)	DNA microarray analysis	66
Photorhabdus luminescens		Repression by LuxS (QS system)	DNA microarray analysis	60
Pseudomonas aeruginosa	HSI-1	RetS/LadS	DNA microarray analysis	75
		RetS/LadS	Western blotting	75
		Gac/Rsm	DNA microarray analysis	14, 15
		Repression by LasR (QS system)	DNA microarray analysis	36
		Repression by LasR (QS system)	RT-PCR	65

Continued on following page

TABLE 1—*Continued*

Organism	Gene cluster	Regulation profile or environmental and/or protein regulator(s)[a]	Analytical technique[b]	Reference(s)
		Repression by MvfR (TF/QS system)	DNA microarrays	36
		Repression by MvfR (TF/QS system)	RT-PCR	65
		RsmA	DNA microarray analysis	14
		RsmA	Reporter fusion	14
		RsmA	EMSA	14
		PpkA/PppA/Fha/TagR		51, 76
	HSI-2	QS (AHL)	DNA microarray analysis	107
		LasR (QS system)	ChIP-to-chip	44
		Induction by LasR/RhlR (QS system)	DNA microarray analysis	96
		Induction by LasR/RhlR (QS system)	DNA microarray analysis	114
		Induction by LasI/RhlI (QS system)	Proteome analysis	2
		Induction by LasR (QS system)	DNA microarray analysis	36
		Induction by LasR (QS system)	RT-PCR	65
		Induction by MvfR (TF/QS system)	DNA microarray analysis	36
		Induction by MvfR (TF/QS system)	RT-PCR	65
		H-NS-like protein MvaT	DNA microarray analysis	24
		Induction by VqsR	DNA microarray analysis	54
		Repression by PsrA (TF)	DNA microarray analysis	58
	HSI-3	Gac/Rsm	DNA microarray analysis	14, 15
		QS (AHL)	DNA microarray analysis	107
		Induction by LasR/RhlR (QS system)	DNA microarray analysis	96
		Induction by LasR (QS system)	DNA microarray analysis	36
		Induction by LasR (QS system)	RT-PCR	65
		Induction by MvfR (TF/QS system)	DNA microarray analysis	36
		Induction by MvfR (TF/QS system)	RT-PCR	65
		PqsE	DNA microarray analysis	36
		H-NS-like protein MvaT	DNA microarray analysis	24
		Induction by epithelial cells	DNA microarray analysis	32
Pseudomonas fluorescens	Pf-5	*In vivo* induction by necrotic roots	DNA microarray analysis	7
		GacA (RR)	DNA microarray analysis	48
Pseudomonas putida	HSI-2 (PP_4071–PP_4085)	H-NS-like protein TurA	DNA microarray analysis	92
Pseudomonas syringae pv. syringae B728a		RetS/LadS/GacS	RT-PCR	91
Rhizobium leguminosarum		Temp	Proteome analysis	11
Salmonella enterica		H-NS	DNA microarray analysis	67
		Induction upon macrophage infection	Reporter fusion	82
		Induction upon macrophage infection	RT-PCR	82
		Repression by SsrB (RR)	Reporter fusion	82
		Repression by SsrB (RR)	RT-PCR	82
Vibrio cholerae	*vas*	Flagellar regulatory proteins	DNA microarray analysis	103
		Flagellar regulatory proteins	RT-PCR	103
		σ^{54}	Mutagenesis	87
		σ^{54}	DNA microarray analysis	103
		Induction by indole (DksA/ppGpp)	DNA microarray analysis	77
		Induction in rabbit intestine	DNA microarray analysis	33
		Induction in rabbit intestine	Northern blotting	33
	hcp	σ^{54}	Western blotting	54
		HapR (QS system)	Western blotting	54
		LuxO (QS system)	Western blotting	54
		cAMP-CRP	Western blotting	54
		Hfq	Western blotting	54
Yersinia pestis	Locus 1 (YPO0499)	Induction at low temp	Proteome analysis	85
		Induction at low temp	Proteome analysis	49
		Induction at low temp	Proteome analysis	31
		Induction at low temp	RT-PCR	93
		Induction at low temp	DNA microarray analysis	29
		Induction at low temp	DNA microarray analysis	47
		Repression at high temp (37°C)	DNA microarray analysis	73
		Induction in human plasma	DNA microarray analysis	29
		Indirect regulation by RovA (MarR-like TF)	DNA microarray analysis	25
		Indirect regulation by RovA (MarR-like TF)	RT-PCR	25
		Indirect regulation by RovA (MarR-like TF)	Reporter fusion	25
		Induction by iron starvation	Proteome analysis	84

[a] RR, response regulator.
[b] RT-PCR, reverse transcription-PCR; IVET, *in vivo* expression technology; FURTA, Fur titration assay; EMSA, electrophoretic mobility shift assay; ChIP-to-chip, chromatin immunoprecipitation (ChIP)-microarray analysis.

σ^{54}. Alternative sigma factors confer transcription specificity by directing the core RNA polymerase to specific promoters. Among the variety of alternative sigma factors, only σ^{54} has been reported to control the expression of T6SS gene clusters. Encoded by the *rpoN* gene, σ^{54} is an alternative sigma factor that recognizes and binds conserved sequences (TGGCA and TGCA) centered at positions −24 and −12 relative to the transcriptional +1 start site (112). Upon recruitment of the RNA polymerase, σ^{54} induces the formation of the closed complex; however, transition to the open complex requires activation that is dependent upon a specific protein, called enhancer binding protein (EBP). This protein carries an N-terminal receiver domain that is phosphorylated under specific environmental conditions. EBPs bind to upstream activating sequences (UAS) located between 100 and 1,000 bp from the σ^{54} binding box and, through DNA looping (which is facilitated by the DNA-bending IHF) and ATP hydrolysis, induce DNA melting at the +1 site of transcription (19, 90, 112). Activation of σ^{54}-dependent promoters thus requires σ^{54}, a cognate EBP, UAS, and IHF. The contribution of σ^{54} and cognate EBPs to T6SS gene cluster regulation is evident in several bacteria, such as *V. cholerae* and *A. hydrophila*. Mutations in the *rpoN* and EBP *vasH* genes in *V. cholerae* were isolated in the same screen that identified the T6SS (i.e., in mutants that resist predation by amoebae) (87). Indeed, regulation by σ^{54} has been confirmed recently by two independent studies with conflicting findings; Ishikawa and colleagues have shown that σ^{54} positively regulates the expression of the *hcp* genes (54), whereas Syed and colleagues reported that σ^{54} negatively regulates the expression of the *vas* genes (103). However, this discrepancy may be due to differences in the strain background, since the strain used in the latter study carries an inactivated *hapR* gene, one of the major positive regulators of *hcp* expression (54). Interestingly, the authors of the study showed that the FliA sigma factor (σ^{28}), involved in the flagellar regulatory cascade, is also a repressor of both the T6SS gene locus and the *hapR* gene (103). Computer analyses of the *V. cholerae hcp* promoter further showed the presence of consensus σ^{54} and IHF binding boxes (54). In *A. hydrophila*, the VasH EBP positively controls the expression of the T6SS gene cluster (101). Several T6SS gene clusters, such as those of *P. atrosepticum*, *Dickeya dadantii*, *Pseudomonas syringae*, *Marinomonas* sp., and *P. aeruginosa* HSI-2 and HSI-3, possess consensus σ^{54} binding sequences and encode EBPs, suggesting that their regulation is dependent upon σ^{54}.

Quorum sensing. QS is a regulatory mechanism dependent upon cell density. In the most common QS mechanism, bacteria produce diffusible signaling molecules such as acyl homoserine lactones (AHL), which accumulate when the cell density increases. When the AHL concentration reaches a given threshold, the whole bacterial population responds homogeneously by the specific activation of target genes (20, 110). This cell-to-cell communication controls multiple cell functions, including pathogenesis. At a molecular level, a protein of the LuxI family synthesizes the AHL, which is in turn recognized by a protein of the LuxR family. The analysis of data from a large number of transcriptional profiling studies reveals that QS is a major regulatory mechanism for type VI secretion gene expression. As examples, the *Photorhabdus luminescens* T6SS gene cluster is downregulated in a *luxS* mutant strain (60) and the *P. atrosepticum* ExpIR system induces the expression of the T6SS genes, as well as of the *hcp* and *vgrG* genes disseminated throughout the chromosome (66). A recent study showed that the *A. hydrophila vas* gene cluster is regulated by the AhyRI QS system (59). In *V. cholerae*, the LuxO QS system regulates the expression of the *hcp* gene (54) and of the HapR transcriptional activator, linking QS and Hcp regulation. In several bacteria, such as *P. aeruginosa* and *B. cenocepacia*, multiple QS systems act in parallel to fine-tune the expression of virulence factors, including T6SS. In *B. cenocepacia*, the T6SS gene cluster is regulated by both the CepRI and the CciRI QS systems, although it is not yet known how these two QS systems are connected and how they each contribute to T6SS regulation (26, 80). The situation in *P. aeruginosa* is even more complicated, especially due to the presence of multiple T6SS gene clusters and the presence of multiple QS systems (Fig. 1): the HSI-1 locus is controlled by the LasRI and MvfR QS systems (36, 65). The LasRI, RhlRI, and MvfR QS systems contribute to the regulation of the HSI-2 and HSI-3 loci (36, 44, 65, 96, 107, 114), whereas the VqsR QS system is restricted to the HSI-2 locus (55).

AraC-, TetR-, and MarR-like transcriptional activators. Transcriptional factors (TFs) are widely distributed in bacteria and fulfill widely diverse functions, including regulation of carbon metabolism, cell maintenance and sporulation, antibiotic resistance or synthesis, and detoxification (104). Several examples of TF-dependent T6SS regulation mechanisms have been described so far. In enteroaggregative *E. coli*, expression of the *sci-2* gene cluster is positively regulated by AggR, an AraC-like TF also inducing the expression of plasmid-carried genes mediating aggregative adherence (37). The AraC-like TF EsrC induces the expression of the *E. tarda evp* gene cluster (116). In *Y. pestis*, the MarR-like TF RovA contributes to the expression of a T6SS gene cluster (25). The *P. aeruginosa* MvfR TF indirectly induces the expression of the HSI-2 and HSI-3 loci and represses the expression of the HSI-1 locus through the control of the production of the hydroxyalkylquinoline (HAQ) QS molecule (36, 65). The *P. aeruginosa* HSI-2 locus is regulated by PsrA, a TetR-like TF which controls fatty acid metabolism and O-antigen biosynthesis (58), allowing reorganization of membrane composition and increased resistance to antimicrobial peptides and antibiotics.

The complexity of regulation by TFs is exemplified in two organisms. In *V. cholerae*, the expression of the *hcp* gene is regulated on one hand by the TetR-like TF HapR working with QS regulators (54) and on the other hand by the HlyU TF (113). In *B. pseudomallei*, multiple TFs control the expression of the *tss5* T6SS gene cluster: the AraC-like BprC TF operates at the end of an activating cascade involving the TetR-like BspR, the OmpR-like BprP, and the AraC-like BsaN TFs (102). A similar TF cascade is probably involved in the regulation of the corresponding *B. mallei* T6SS gene cluster, even though only the AraC-like TF BMAA1517 has been shown to activate its expression (95).

Fur. The ferric uptake regulator Fur is the main repressor controlling the expression of genes involved in iron uptake and resistance to acidic stress. In the presence of iron, the Fe-Fur complex binds to specific Fur boxes that usually overlap with transcription consensus sites, blocking access to the RNA polymerase. Upon iron starvation, Fe dissociates from Fur, induc-

ing the release of Fur from the promoter and subsequent gene expression (21). Several T6SS gene clusters have been reported to be regulated by iron (see the above section), and their expression is dependent upon Fur. This is the case for the *E. tarda* T6SS gene cluster (109), the enteroaggregative *E. coli sci-1* locus (Brunet and Cascales, unpublished), and the FPI-carried T6SS cluster in *F. tularensis* (18, 35).

Two-component systems. TCS represent one type of major signaling pathway in bacteria. They couple the sensing of an environmental signal to an adaptative response. Generally, TCS are composed of two subunits tightly linked: the sensor kinase transduces a signal to the response regulator through phosphate transfer (phosphorelay). Phosphorylation-dependent activation of the response regulator then determines the output and the subsequent expression of specific genes (reference 12 and linked reviews; 71). The output domain of the response regulator is often a DNA binding domain which binds to specific sequences and regulates the expression of genes involved in most of the physiological, adaptative, and virulence pathways (42, 43). TCS thus constitute the simplest and the most sensitive and efficient regulatory mechanism for a fast response in changing environments. However, the nature or identity of the signal is most of the time completely unknown. Because T6SS gene clusters are usually regulated by environmental conditions such as the presence of host cells, it is not surprising that TCS are the most common regulatory mechanisms. Indeed, the EsrAB TCS induces the expression of the *evp* gene cluster in *E. tarda* (89, 109, 116). In *B. mallei*, the Tss1 locus is upregulated by the VirAG TCS (95). In *S. enterica*, the *sci* T6SS gene cluster is controlled by the SsrAB (SpiR/SsrB) TCS, one of the major regulatory pathways of *Salmonella* virulence (82). SsrAB is a TCS encoded within *Salmonella* pathogenicity island 2 (SPI-2), and the response regulator SsrB is able to bind to most, if not all, SPI-2 promoters, including that of the type III secretion system (T3SS) gene cluster. As a consequence, the T3SS is positively controlled by SsrAB (40). In contrast, SsrAB exerts negative control of the *sci* T6SS gene cluster (82). Thus, the T3SS and T6SS are inversely regulated in *S. enterica*. In *Francisella* spp., the FPI cluster is regulated by the histidine kinase KdpD and PmrA, an orphan response regulator required for antimicrobial peptide resistance through modification of the outer membrane properties (9, 72).

POSTTRANSCRIPTIONAL REGULATION: MODULATION OF T6SS mRNA LEVELS

In *P. aeruginosa*, a pair of sensor kinases, RetS and LadS, regulates the expression of the HSI-1 T6SS gene cluster (75). The signals sensed by the RetS and LadS proteins are still unknown, but RetS and LadS control the transition of *P. aeruginosa* from acute infection to chronic persistence (and *vice versa*) (45, 106). Thanks to their actions, the T3SS and T6SS are inversely regulated: RetS is an activator of the expression of T3SS genes but represses genes involved in biofilm formation and T6SS expression. LadS has reciprocal effects. This regulation mechanism suggests that the HSI-1 T6SS favors the chronic phase of infection (75).

RetS and LadS are not linked to a typical response regulator but modulate the phosphorylation status of the GacS sensor kinase of the GacSA TCS. Phosphotranfer to GacA promotes the transcription of the small regulatory RNAs *rsmZ* and *rsmY* (61). *rsmZ* and *rsmY* titrate and sequester the RNA binding protein RsmA, a protein which acts as a translational repressor of target genes by hindering access of the ribosome and accelerating mRNA decay. Hence, *rsmZ* and *rsmY* relieve RsmA repression, allowing the translation of RsmA-targeted mRNA, including the HSI-1 T6SS mRNA (14). Accordingly, RsmA is a translational repressor of the HSI-1 mRNAs whereas GacSA and *rsm* function as activators of the HSI-1 locus. Besides its role in the upregulation of the HSI-1 locus, the Gac/Rsm pathway has been reported recently to downregulate the expression of the *P. aeruginosa* HSI-3 T6SS cluster (14, 15) (Fig. 1). A probably similar regulatory pathway controls the expression of the *P. syringae* pv. syringae and *P. fluorescens* Pf-5 T6SS gene clusters (48, 91). Interestingly, an orphan sensor of the RetS family, AtsR, has been reported to repress the expression of the *B. cenocepacia* T6SS gene cluster (5).

COMPLEX NETWORKS OF REGULATION AND REGULATION OF MULTIPLE CLUSTERS

From the data collected so far, it is clear that T6SS gene clusters are subjected to complex regulatory networks. Three different cases are summarized below. The clusters in these cases are regulated by distinct networks and cascades, and one of the current challenges is to understand how these multiple regulatory pathways converge or are connected. Another dimension in the regulation mechanisms is generated by the presence of multiple T6SS gene clusters in a single bacterium. Most genomes carry one, two, or three T6SS gene clusters, whereas several bacteria such as *Yersinia* species and *B. mallei* and *B. pseudomallei* bear as many as five to seven copies (10). None of the studies so far have dissected how bacteria juggle the regulatory networks, how the different cascades are coordinated or connected, or how the different T6SS machines engage in cross talk at the regulatory level.

***P. aeruginosa* HSI clusters.** The *P. aeruginosa* HSI-1 gene cluster is repressed at the transcriptional level by QS and posttranscriptionally regulated by the Gac/Rsm pathway (Fig. 1). Despite the fact that only the RetS-like protein AtsR and the Cep/Cci QS systems have been shown to regulate the *B. cenocepacia* T6SS gene cluster, one may hypothesize that this cluster has a similar regulatory network. Different QS pathways also control the expression of the *P. aeruginosa* HSI-2 and HSI-3 gene clusters, but further studies need to decipher how QS regulators coordinate their efforts. The presence of two clusters encoding specific σ^{54}-dependent EBPs raises the question of whether the HSI-2 and HSI-3 EBPs have independent, overlapping, or conflicting actions.

***V. cholerae hcp*.** A comprehensive study of the regulation of the *V. cholerae hcp* gene has been reported by Ishikawa and coworkers. They revealed that *hcp* is regulated by the alternative sigma factor σ^{54}, the global regulator cyclic AMP (cAMP) receptor protein (CRP), and the HapR TF. The activation of HapR is at the end of a regulatory cascade involving the small RNA binding protein Hfq, the LuxS and CqsA AHL synthesizers, and the LuxO QS regulator (54). Although the contribution of these regulators in modulating the expression of the *vas* cluster has not been tested yet, evidence for regulation of the T6SS main cluster by σ^{54}, HapR, and the flagellar regula-

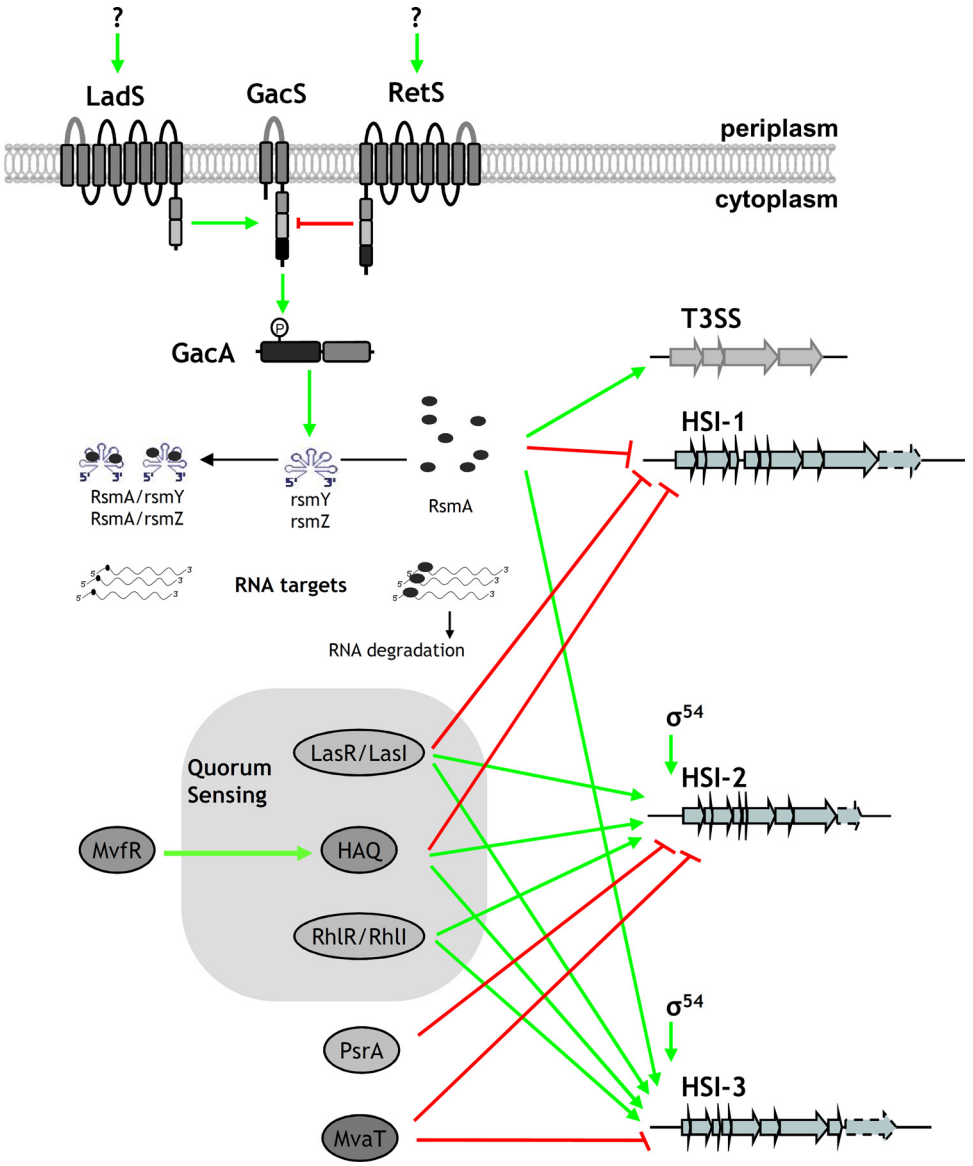

FIG. 1. Regulation of the *P. aeruginosa* T6SS loci. The various regulatory pathways involved in the regulation of *P. aeruginosa* HSI-1, HSI-2, and HSI-3 T6SS clusters are indicated. Effects of induction (green arrows) and repression (red bars) are shown. The predicted σ^{54}-dependent regulation suggested by *in silico* analyses is depicted. The signal(s) that leads to LadS or RetS activation is unknown and is thus represented by a question mark.

tory cascade has been reported (103). A role for the indole-responsive regulatory pathway has also been suggested recently (77).

FPI of *Francisella* spp. The T6SS gene cluster carried within the FPI (Fig. 2) exploits the most complex regulatory network described so far: the orphan response regulator PmrA is phosphorylated by the KdpD histidine kinase and recruits the MglA and SspA proteins to initiate FPI gene transcription (9). MglA and SspA are two proteins which associate with the RNA polymerase and with a fifth actor, PigR (27). The interaction of PigR with the MglA/SspA complex is dependent upon a signaling molecule, the stringent response alarmone ppGpp (27). The regulatory network also involves several proteins such as Fur, FevR, MigR, RipA, KdpE, FTN1453, and the small RNA binding protein Hfq (Table 1), although their contributions to FPI regulation and their coordination with the PmrA regulatory pathway have not been characterized yet.

COREGULATION OF T6SS GENE CLUSTERS WITH OTHER VIRULENCE DETERMINANTS AND REGULATORY CROSS TALK AMONG T6SS AND OTHER VIRULENCE DETERMINANTS

The T6SS gene clusters should be coordinately regulated with other virulence factors to be expressed at the appropriate time during the infection process. It is easily conceivable that global regulatory mechanisms such as QS coordinate the co-

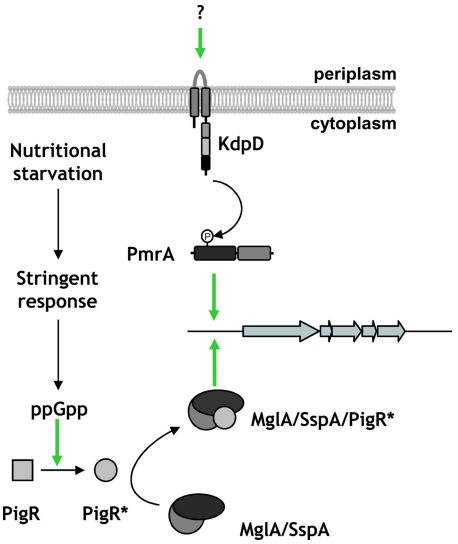

FIG. 2. Regulation of the FPI T6SS locus. Expression of the locus is dictated through RNA polymerase recruitment by the phosphorylated PmrA protein and the MglA/SspA/PigR complex. The form of PigR activated by the stringent response through the alarmone ppGpp is indicated by an asterisk. The signal that leads to KdpD-dependent PmrA phosphorylation (?) is unknown.

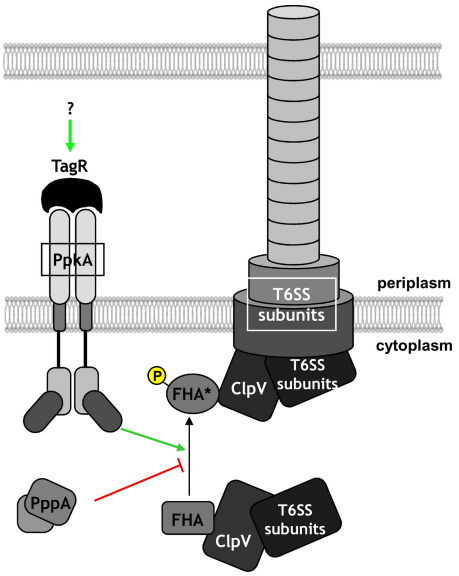

FIG. 3. Posttranslational regulation. PpkA dimerization induced by the TagR protein leads to FHA protein phosphorylation and subsequent recruitment of essential components of the secretion apparatus. PpkA activity is antagonized by the activity of the PppA phosphatase. Mechanisms of activation (green arrows) and inhibition (red bars) are indicated. The asterisk denotes activated FHA protein.

expression of virulence determinants; this is the case for the type II secretion system (T2SS) and the various T6SS gene clusters in *P. aeruginosa* and *B. cenocepacia* (65, 80, 96, 107). In *B. pseudomallei*, the T3SS and T6SS are coregulated by a cascade of TFs: the main activator of the T3SS, the AraC-like TF BsaN, regulates the expression of BprC, which turns on the T6SS gene cluster (102); however, in several bacteria, such as *S. enterica* and *P. aeruginosa*, the T6SS and T3SS are inversely regulated, allowing the transition between the chronic and acute phases of infection (75, 82). In these cases, T6SS are usually coregulated with genes that promote the chronic phase and implantation (e.g., those involved in fitness, stress sensing, and biofilm formation) rather than infection. In *V. cholerae*, the cascade regulating the T6SS *vas* locus is partly redundant with the flagellar regulatory cascade (103). In *Y. pestis*, the RovA TF activates the T6SS gene cluster, as well as invasin, an invasion factor which mediates translocation across the intestinal epithelium (25). It is noteworthy that no study reported regulatory cross talk between the type VI secretion machine itself and virulence genes or between T6SS when they coexist in a single bacterium, as has been shown for the *P. aeruginosa* T3SS and flagellum (98).

POSTTRANSLATIONAL ACTIVATION OF TYPE VI SECRETION MACHINES

T6SS are also activated at the posttranslational level through a yet unknown signal. This posttranslational activation mechanism is therefore a further level of regulation, allowing the T6SS to respond rapidly and reversibly to specific stimuli. In two important recent studies, Mougous and colleagues sketched out the molecular bases of this exciting mechanism (51, 76): it implies the existence of a couple consisting of a serine-threonine kinase (Stk) and a phosphatase (Stp) which influences the phosphorylation status of an FHA protein (76) (Fig. 3). FHA proteins are involved in phosphorylation-dependent protein-protein interaction and signaling (81). Although Stk, Stp, and FHA proteins are shared by several T6SS gene cluster regulatory networks, only the *P. aeruginosa* HSI-1-associated posttranslational mechanism has been characterized so far. The current model proposes that the periplasmic TagR protein senses an unknown signal and induces the signaling cascade through dimerization of the membrane-associated Hanks-type threonine kinase PpkA (51). PpkA autophosphorylates at two threonine residues (74) and then transfers the phosphate group onto the FHA protein (51). The FHA protein interacts with a core component of the T6SS, the ClpV ATPase, and the FHA protein-ClpV complex is recruited to the secretion apparatus upon PpkA-dependent FHA protein phosphorylation (51). The combination of PpkA action as a docking or scaffolding protein at the T6SS and FHA protein phosphorylation leads to the allosteric activation of ClpV ATP hydrolysis activity, allowing machine assembly or substrate secretion. The serine-threonine phosphatase PppA antagonizes the role of PpkA through FHA protein dephosphorylation and subsequent machine disassembly (76) (Fig. 3). However, several T6SS do not possess this activation mechanism and yet are active. The ATPase activity of ClpV may thus be constitutively activated or modulated by other factors.

CONCLUDING REMARKS

Much progress has been made in understanding T6SS regulation in recent years. As highlighted in this review, broadly diverse regulatory mechanisms have been exploited or hijacked by T6SS; however, much more should be done to explore these different mechanisms and to define the cross talk that takes

place among T6SS gene clusters and other virulence determinants. Characterizing these regulatory networks, defining how they are coordinated, and identifying the environmental signals sensed by these systems are critical to determining the role of these gene clusters but may also help to identify the secreted substrates.

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