Integrating signals to drive T6SS killing

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The type VI secretion system (T6SS) is a nanoweapon used by bacteria to defend from predators, subvert eukaryotic cells and fight other microbes including bacterial competitors and fungi (Allsopp *et al.*, 2020). Functioning like a spring-loaded harpoon, bacteria fire this system directly into target cells directly delivering a toxic payload for bacterial gain (Allsopp *et al.*, 2020). Identified in more than 25% of Gram-negative bacteria and containing 13 core components, the T6SS has found widespread use in both environmental and pathogenic organisms (Allsopp *et al.*, 2020). The core components are typically encoded in one large operon but orphan gene clusters encoding additional components are common. For instance, *Vibrio cholerae* strains contain one large central cluster and between 2 to 5 auxiliary (or orphan) islands (recently reviewed in Crisan and Hammer, 2020).

The high conservation of the large clusters encoding the T6SS and orphan islands indicates that these systems are expressed and confer a selective advantage to the bacteria. However, the expression, assembly and secretion of the many proteins required to form a functional T6SS is energetically costly and may not be necessary or favourable for all environments and/or conditions encountered by a bacterium (Basler, 2015). Indeed, unless resources are limited or cell density is high it makes little sense to expend resources being highly aggressive (Gonzalez and Mavridou, 2019). Thus, expression of the T6SS is highly regulated in many organisms, presumably to enable expression in defined conditions (Silverman *et al.*, 2012; Allsopp *et al.*, 2017; Wang *et al.*, 2019).

Control of the T6SS occurs at multiple levels with complex transcriptional (e.g. VasH), post-transcriptional (e.g. RsmA) and post-translational (e.g. PpkA/Fha) regulatory mechanisms present in bacteria (Figure 1) (Silverman *et al.*, 2012; Allsopp *et al.*, 2017; Wang *et al.*, 2019). Many T6SS genes are not expressed well in laboratory conditions, however a common theme is enhanced expression in the later stages of growth, nutrient availability and upon surface contact (Silverman *et al.*, 2012; Wang *et al.*, 2019). Moreover, different bacteria have integrated T6SS genes into a variety of existing regulatory networks enabling expression under beneficial conditions. Major drivers of the T6SS are; Quorum sensing, salinity, osmolality, metal iron availability and stress (Silverman *et al.*, 2012; Wang *et al.*, 2019; Crisan and Hammer, 2020). Some of these conditions are sensed by bacteria via two-component systems and relayed into transcriptional regulators for direct control of T6SS genes. However, others act indirectly and for most of them the mechanism is still unclear or complex with multiple regulators at play.

Regardless of the regulators at play, transcription is the first critical step and requires RNA polymerase for mRNA synthesis to occur. Sigma factors specify transcription by binding to characteristic promoter sequences, recruiting the RNA polymerase and enabling the formation of the open complex (Browning and Busby, 2004). The expression of the majority of genes is controlled by the 'housekeeping' sigma factor 70 (also known as RpoD, σ^{70} σ^{A}) (Browning and Busby, 2004). However, the major alternative sigma factor, RpoN (Sigma 54, σ^{54} and σ^{N}), has been implicated in modulating the T6SS in many Gram-negative bacteria (Bernard *et al.*, 2011; Silverman *et al.*, 2012; Crisan and Hammer, 2020; Seibt *et al.*, 2020).

RpoN was originally discovered to be a central player in controlling nitrogen metabolism (Francke *et al.*, 2011). It is also a key factor driving flagella biosynthesis and motility

(Francke *et al.*, 2011). More recently, this regulator has been shown to be a general regulator of virulence, and numerous bacterial cell envelope and surface components (Francke *et al.*, 2011). Unlike Sigma 70, RpoN requires an enhancer binding protein (EBP) for activation. EBPs typically bind within 100-200 bp of an RpoN binding site; DNA bending via integration host factor (IHF) enables the interaction between the EBP and RpoN leading to open complex formation and transcription (Schumacher *et al.*, 2006). Without the EBP, RpoN binds and prevents transcription from a locus (Schaefer *et al.*, 2015). EBPs are typically comprised of three domains; an N-terminal regulatory domain, an AAA+-family ATPase domain, and a C-terminal Helix-turn-Helix DNA-binding domain that recognises the -24 and -12 promoter elements (Schumacher *et al.*, 2006). Variability in the N-terminal and C-terminal domains amongst EBPs enable a diverse range of signals to be detected and distinct DNA sequences to be bound (Francke *et al.*, 2011). Therefore, influencing the availability of EBPs and their stimuli enable the bacteria to respond to their environment by modulating different surface components including the T6SS.

RpoN and EBPs are involved in the regulation of T6SS genes in *Vibrio cholerae, Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* amongst others (Bernard *et al.*, 2011; Sana *et al.*, 2013; Storey *et al.*, 2020). In most cases, RpoN is critical for the expression of the T6SS, but it has been reported to play an indirect repressive role in the regulation of the H2-T6SS of *P. aeruginosa* (Bernard *et al.*, 2011; Sana *et al.*, 2013; Storey *et al.*, 2020). Interestingly, most EBPs associated with the T6SS are encoded within the central T6SS clusters (Bernard *et al.*, 2011; Sana *et al.*, 2013). This is the case for Sfa2 and Sfa3, two RpoN enhancer proteins of *P. aeruginosa* that are encoded within the H2- and H3-T6SS clusters, respectively (Sana *et al.*, 2013). Similarly, PSPTO_2549 and PSPTO_5424, found within the HIS-I and HIS-II T6SS clusters of *P. syringae* pv. tomato, encoded two putative σ^{54} transcriptional regulators (Chien *et al.*, 2020) with 57% and 71% identity to Sfa2. Certainly, the best characterised T6SS-related EBP is VasH, a σ^{54} activator necessary for functional T6SSs in *Aeromonas hydrophila*, *Vibrio fischeri*, *Pectobacterium atrosepticum, Marinomonas* sp. and most importantly *V. cholerae* (Bernard *et al.*, 2011; Kitaoka *et al.*, 2011; Guckes *et al.*, 2020).

In *V. cholerae* strain O1 El Tor A1552, an isolate that causes cholera disease, *vasH* is encoded within the central cluster together with the majority of the system components (Pukatzki *et al.*, 2006). VasH is required for the RpoN-dependent transcription of two orphan islands in this strain (Kitaoka *et al.*, 2011). These orphan operons contain the *hcp* and *vgrG* genes, which encode for the tube and the tip proteins of the system, two vital core components required for a functional T6SS (Zheng *et al.*, 2011). Interestingly, in A1552 the production of Hcp and VgrG is only detected under low temperature and high osmolarity conditions whilst the structural cluster has a higher transcription rate (Ishikawa *et al.*, 2012). Recently, the VasH-dependent molecular mechanism integrating the environmental cues that co-regulate the structural and the orphan clusters of A1552 have been explored by Seibt *et al.* (2020).

Since the activity of EBPs are normally controlled by their N-terminal domains (Schumacher *et al.*, 2006; Francke *et al.*, 2011), *Seibt et al.* expressed a $VasH^{A1552}$ truncated version lacking the N-terminal regulatory domain which resulted in a constitutively-active T6SS that was independent of temperature and osmolarity (Seibt *et al.*, 2020). In line with a previous report, $VasH^{A1552}$ was not necessary for the expression

of the central large structural operon within which it is encoded (Dong and Mekalanos, 2012). Rather, VasH acts to bind the promoters and switch-on expression of the two *hcp* and *vgrG* orphan clusters in conjunction with RpoN and IHF (Seibt *et al.*, 2020). Thus, the environmental cues regulating the structural cluster are integrated into the expression of the orphan clusters through VasH, in a sophisticated mechanism of cascading regulation. In this mechanism, VasH acts as a gatekeeper. VasH is expressed with the main cluster under conditions of low temperature and high osmolarity. Once VasH levels reached those required to activate transcription, the gate is opened and expression of the orphan operons occurs. This results in production of the missing core components of the T6SS (Hcp and VgrG) leading to a functional and finely tuned apparatus.

Contrary to the complexity of the T6SS regulation in A1552, the less virulent V52, which causes gastrointestinal irregularities, displays constitutive and signal independent expression of the T6 system (Pukatzki *et al.*, 2006). This means that strains of *V. cholerae* with different degrees of virulence in humans have distinct T6SS expression strategies with V52 being offensive and continuously expending energy firing its T6SS, whilst A1552 opts for a defensive strategy. This example illustrates how bacteria use a sophisticated regulatory network to integrate signalling cues from the environment and orchestrate a defensive plan when necessary.

In other bacteria, the environmental cues regulating T6SSs are known, although the fine regulatory network behind it may not be clear. For instance, the T6SS of *A. tumefaciens* is induced under acidic pH conditions like those encountered by the bacterium upon leaf wounding and in the apoplast when they penetrate the plant (Wu *et al.*, 2012). The T6SSs of opportunistic enteric pathogen *Edwardsiella tarda* and enteroaggregative *E. coli* (EAEC) are repressed in the presence of iron through the action of the regulator Fur (Bernard *et al.*, 2011). Some bacteria can also sense killing of their kin, such as *P. aeruginosa* that recognises specific signals released from lysed *Pseudomonas* to activate a danger sensing mechanism that promotes T6SS production through the GacA/S cascade (Le Roux *et al.*, 2015). In other cases, bacteria respond to cues from the host environment. For example, *V. cholerae* induces the T6SS in the presence of chitin, the main component of the crustaceans exoesqueletons they colonise, killing competitors on the host and even up taking their DNA (Borgeaud *et al*., 2015). Similarly, *V. fischeri* upregulates the expression of the T6SS upon colonisation of the squid light organ to be the sole resident (Speare *et al.*, 2018). In a few cases, the specific host signal has been identified *e.g.* the mammalian mucin from gut secretion activates T6SS in *V. cholerae* and allows this strain to fight microbiome competitors (Bachmann *et al.*, 2015). In a similar strategy, the T6SS of *Klebsiella pneumoniae* is transcriptionally induced by polymyxin antibiotics and the antimicrobial peptide human β-defensin 3 via the action of the two-component system PhoPQ (Storey *et al.*, 2020). Thus, bacteria can ‘wire-up’ the regulator control of the T6SS to respond to their environment optimally.

T6SS regulation can be highly complex with multiple layers of control to secure an optimal balance between defence, aggression and energy expenditure. The number of environmental cues responsible for controlling the T6SSs is continuously growing but for many of the systems they are still unknown. The integration of environmental cues through VasH is a formidable example of successful evolution of a control mechanism for an “expensive” but advantageous system to drive the killing of a bacterium’s competitors.

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References

Allsopp, L.P., Bernal, P., Nolan, L.M., and Filloux, A. (2020) Causalities of war: The connection between type VI secretion system and microbiota. *Cell Microbiol* **22**: 1–9.

Allsopp, L.P., Wood, T.E., Howard, S.A., Maggiorelli, F., Nolan, L.M., Wettstadt, S., and Filloux, A. (2017) RsmA and AmrZ orchestrate the assembly of all three type VI secretion systems in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **114**: 7707–7712.

Bachmann, V., Kostiuk, B., Unterweger, D., Diaz-Satizabal, L., Ogg, S., and Pukatzki, S. (2015) Bile salts modulate the mucin-activated type VI secretion system of pandemic *Vibrio cholerae*. *PLoS Negl Trop Dis* **9**: 1–22.

Basler, M. (2015) Type VI secretion system: Secretion by a contractile nanomachine. *Philos Trans R Soc B Biol Sci* **370**: 20150021.

Bernard, C.S., Brunet, Y.R., Gavioli, M., Lloubès, R., and Cascales, E. (2011) Regulation of type VI Secretion gene clusters by σ54 and cognate enhancer binding proteins. *J Bacteriol* **193**: 2158–2167.

Browning, D.F. and Busby, S.J.W. (2004) The regulation of bacterial transcription initiation. *Nat Rev Microbiol* **2**: 57–65.

Borgeaud, S., Metzger, L.C., Scrignari, T., Blokesch, M. (2015) The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* **347**: 63-67.

Chien, C.F., Liu, C.Y., Lu, Y.Y., Sung, Y.H., Chen, K.Y., and Lin, N.C. (2020) HSI-II Gene Cluster of *Pseudomonas syringae* pv. tomato DC3000 Encodes a Functional Type VI Secretion System Required for Interbacterial Competition. *Front Microbiol* **11**: 1–14.

Crisan, C. V. and Hammer, B.K. (2020) The *Vibrio cholerae* type VI secretion system: toxins, regulators and consequences. *Environ Microbiol* doi:10.1111/1462-2920.14976.

Dong, T.G. and Mekalanos, J.J. (2012) Characterization of the RpoN regulon reveals differential regulation of T6SS and new flagellar operons in *Vibrio cholerae* O37 strain V52. *Nucleic Acids Res* **40**: 7766–7775.

Francke, C., Groot Kormelink, T., Hagemeijer, Y., Overmars, L., Sluijter, V., Moezelaar, R., and Siezen, R.J. (2011) Comparative analyses imply that the enigmatic sigma factor 54 is a central controller of the bacterial exterior. *BMC Genomics* **12**: 385.

Gonzalez, D. and Mavridou, D.A.I. (2019) Making the Best of Aggression: The Many Dimensions of Bacterial Toxin Regulation. *Trends Microbiol* **27**: 897–905.

Guckes, K.R., Cecere, A.G., Williams, A.L., McNeil, A.E., and Miyashiro, T. (2020) The bacterial enhancer binding protein VasH promotes expression of a type VI secretion system in *Vibrio fischeri* during symbiosis. *J Bacteriol* **202**: 1–15.

Ishikawa, T., Sabharwal, D., Bröms, J., Milton, D.L., Sjöstedt, A., Uhlin, B.E., and Wai, S.N. (2012) Pathoadaptive conditional regulation of the type VI secretion system in *Vibrio cholerae* O1 strains. *Infect Immun* **80**: 575–584.

Kitaoka, M., Miyata, S.T., Brooks, T.M., Unterweger, D., and Pukatzki, S. (2011) VasH

is a transcriptional regulator of the type VI secretion system functional in endemic and pandemic *Vibrio cholerae*. *J Bacteriol* **193**: 6471–6482.

Pukatzki, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., et al. (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci U S A* **103**: 1528–1533.

Le Roux, M., Kirkpatrick, R.L., Montauti, E.I., Tran, B.Q., Brook Peterson, S., Harding, B.N., et al. (2015) Kin cell lysis is a danger signal that activates antibacterial pathways of *Pseudomonas aeruginosa*. *Elife* **2015**: 1–65.

Sana, T.G., Soscia, C., Tonglet, C.M., Garvis, S., and Bleves, S. (2013) Divergent Control of Two Type VI Secretion Systems by RpoN in *Pseudomonas aeruginosa*. *PLoS One* **8**: 1–10.

Schaefer, J., Engl, C., Zhang, N., Lawton, E., and Buck, M. (2015) Genome wide interactions of wild-type and activator bypass forms of σ54. *Nucleic Acids Res* **43**: 7280–7291.

Schumacher, J., Joly, N., Rappas, M., Zhang, X., and Buck, M. (2006) Structures and organisation of AAA+ enhancer binding proteins in transcriptional activation. *J Struct Biol* **156**: 190–199.

Seibt, H., Aung, K.M., Ishikawa, T., Sjöström, A., Gullberg, M., Atkinson, G.C., et al. (2020) Elevated levels of VCA0117 (VasH) in response to external signals activate the type VI secretion system of *Vibrio cholerae* O1 El Tor A1552. *Environ Microbiol* doi:10.1111/1462-2920.15141.

Silverman, J.M., Brunet, Y.R., Cascales, E., and Mougous, J.D. (2012) Structure and regulation of the type VI secretion system. *Annu Rev Microbiol* **66**: 453–472.

Speare, L., Cecere, A.G., Guckes, K.R., Smith, S., Wollenberg, M.S., Mandel, M.J., et al. (2018) Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. *Proc Natl Acad Sci U S A* **115**: E8528–E8537.

Storey, D., McNally, A., Åstrand, M., Santos, J.P.G., Rodriguez-Escudero, I., Elmore, B., et al. (2020) *Klebsiella pneumoniae* type VI secretion system-mediated microbial competition is PhoPQ controlled and reactive oxygen species dependent. *PLoS Pathog* **16**: e1007969.

Wang, J., Brodmann, M., and Basler, M. (2019) Assembly and subcellular localization of bacterial type VI secretion systems. *Annu Rev Microbiol* **73**: 621–638.

Wu, C.F., Lin, J.S., Shaw, G.C., and Lai, E.M. (2012) Acid-Induced Type VI Secretion System Is Regulated by ExoR-ChvG/ChvI Signaling Cascade in *Agrobacterium tumefaciens*. *PLoS Pathog* **8**: e1002938.

Zheng, J., Ho, B., and Mekalanos, J.J. (2011) Genetic analysis of anti-amoebae and anti-bacterial activities of the type vi secretion system in *Vibrio cholerae*. *PLoS One* **6**: e23876.

Figure Legends:

Figure 1: Environmental sensing influences the expression of the T6SS at the transcriptional, post-transcriptional and post-translational levels.

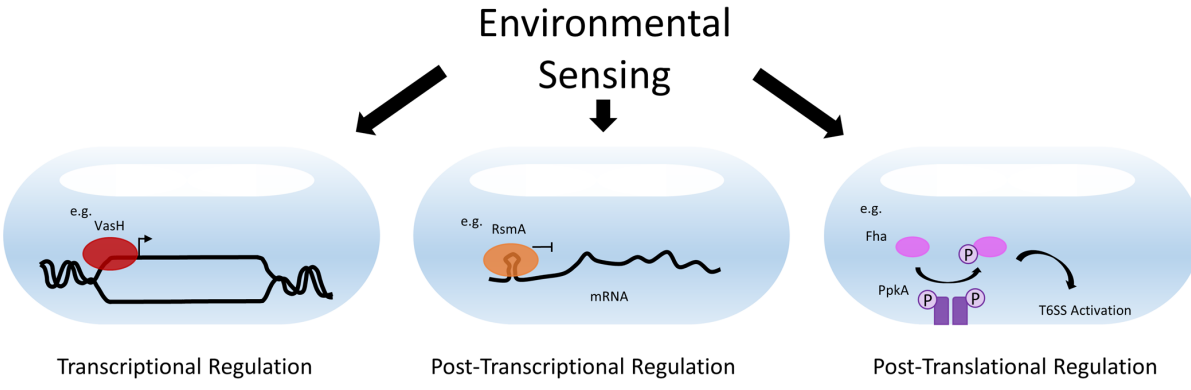
Environmental Sensing
e.g.
VasH
e.g.
RsmA
mRNA
e.g.
Fha
PpkA
P
P
P
T6SS Activation
Transcriptional Regulation
Post-Transcriptional Regulation
Post-Translational Regulation

Figure 1