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Phosphate responsive regulation provides insights for ESX-5 function in *Mycobacterium tuberculosis*

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Abstract

Pathogenic microbes commonly respond to environmental cues in the host by activating specialized protein secretion systems. *Mycobacterium tuberculosis* uses the specialized Type VII ESX protein secretion systems to transport a subset of effector proteins. The ESX-5 secretion system is involved in virulence, but both the mechanism of regulation and activating signal were unknown. Our work, reviewed here, has established that the phosphate sensing Pst/SenX3-RegX3 system directly activates ESX-5 secretion in response to phosphate limitation, a relevant environmental signal likely encountered by *M. tuberculosis* in the host. This review focuses on how elucidation of the ESX-5 regulatory network provides insight into its biological roles, which may include both phosphate acquisition and pathogenesis.

Keywords

ESX secretion; Type VII secretion; Pst system; RegX3

Pathogenic microorganisms often activate specialized protein secretion systems in response to host cues to promote a productive infection. *Mycobacterium tuberculosis* is one of the oldest and most prolific bacterial pathogens in human history, causing the disease tuberculosis. *M. tuberculosis* is a facultative intracellular pathogen that persists within macrophage phagosomes by deploying secreted effector proteins to counteract host defenses, including factors that inhibit fusion with lysosomes to prevent phagosome acidification (Russell 2011). *M. tuberculosis* uses four types of secretion pathways to transport proteins through the complex architecture of its cell envelope: the ubiquitous Sec system, the Tat export system, the accessory SecA2 system and the Type VII ESX systems (Ligon et al. 2012; van der Woude et al. 2013). There are 5 ESX systems, designated ESX-1 to ESX-5. Though the ESX systems each contain conserved core components of the secretion machinery, they appear to have evolved quite divergent functions (Stoop et al. 2012). Identifying regulatory networks that control activity of the ESX secretion systems has been a critical step towards determining their functions. The regulatory mechanism and

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precise function of ESX-5 were previously known. The recent discovery that a phosphate-sensing regulatory system controls ESX-5 activity has provided some insight into its potential roles in *M. tuberculosis* virulence.

The connection between regulation and function is clearly illustrated by the ESX-3 secretion system. ESX-3 is essential for viability of *M. tuberculosis* in standard *in vitro* culture medium (Sassetti & Rubin 2003; Serafini et al. 2009), which frustrated efforts to determine its function. The regulatory mechanisms controlling ESX-3 expression were discovered first, and provided important clues about its function. Two transcriptional regulators, IdeR and Zur, repress ESX-3 when iron or zinc, respectively, is abundant (Rodriguez et al. 2002; Maciag et al. 2007). These observations enabled characterization of ESX-3 function in iron uptake and zinc homeostasis in *M. tuberculosis* (Siegrist et al. 2009; Serafini et al. 2009; Serafini et al. 2013).

ESX-1 was the first of the *M. tuberculosis* ESX secretion systems to be discovered and has a well-established role in virulence. One virulence-associated function of ESX-1 is to permeabilize phagosomes through the secretion of effector proteins, allowing mycobacterial DNA to enter the host cell cytosol (Manzanillo et al. 2012). Given its important role in *M. tuberculosis* pathogenesis, the regulation of ESX-1 has also been intensively investigated. Most ESX-1 regulators act the transcriptional level. EspR controls ESX-1 secretion through transcriptional regulation of the e*spACD* operon, which encodes several proteins that promote secretion of the canonical ESX-1 substrates EsxA (ESAT-6) and EsxB (CFP-10) (Chen et al. 2012; Fortune et al. 2005; Millington et al. 2011; MacGurn et al. 2005; Raghavan et al. 2008). ESX-1 is also regulated by a pair of two-component systems; MprAB represses the *espACD* operon (Pang et al. 2013) while PhoPR positively regulates transcription of many genes in the *esx-1* locus (Gonzalo-Asensio et al. 2008). Several factors activate MprAB, including cell envelope stress and nutrient starvation (Betts et al. 2002; He et al. 2006; Pang et al. 2007), while PhoPR is activated by acidic pH (Baker et al. 2014). The acidic pH signal that activates PhoPR to promote *esx-1* gene expression is encountered by *M. tuberculosis* within the phagosomal environment. This provides a clear example of a niche-specific bacterial response, based on a relevant host signal, which enables the bacterium to counter host defenses.

The function of ESX-5, found only the slow-growing mycobacteria, which include most pathogenic species, remains poorly characterized. As for the ESX-3 system, several components of ESX-5 are essential for viability of *M. tuberculosis in vitro* (Bottai et al. 2012; Di Luca et al. 2012). However, disrupting secretion of ESX-5 substrates results in attenuation *in vivo*, suggesting a significant role for ESX-5 in *M. tuberculosis* pathogenesis mediated by secretion of its effector proteins (Bottai et al. 2012). In the closely related pathogenic species *M. marinum,* most PE and PPE proteins are transported through ESX-5 (Abdallah et al. 2009), and there is evidence that the *M. tuberculosis* ESX-5 also secretes many of these proteins (Sayes et al. 2012). PE and PPE are classes of proteins unique to mycobacteria, and are so named for the characteristic N-terminal proline-glutamic acid (PE) or proline-proline-glutamic acid (PPE) domains (Cole et al. 1998). PE and PPE proteins, which can be either cell-associated or freely secreted, play a diverse array of roles, some of which have been linked to *M. tuberculosis* virulence (Sampson 2011; Fishbein et al. 2015).

Deletion of a subset of *pe* and *ppe* genes encoded within the *M. tuberculosis esx-5* locus causes attenuation in both macrophages and mice (Sayes et al. 2012; Bottai et al. 2012). Other PE and PPE proteins have been implicated in counteracting host defenses or modulating immune responses. PPE2 is thought to inhibit nitric oxide production in activated macrophages (Bhat et al. 2013). PPE18 binds TLR-2 on macrophages, leading to downregulation of protective Th1 proinflammatory cytokines and skewing the host towards the less effective Th2 response (Bhat et al. 2012; Nair et al. 2011; Nair et al. 2009). Finally, PE and PPE proteins are highly antigenic, inducing T-cell immunogenicity *in vivo* (Sayes et al. 2012). Though the precise repertoire of ESX-5-secreted PE and PPE proteins remains to be determined, given their immunogenic nature and external localization, ESX-5 substrates seem well positioned to have substantial interaction with the host during infection. Knowledge of ESX-5 regulation may lead to further discoveries concerning its precise function in *M tuberculosis* physiology and pathogenesis.

In a recent publication, we not only uncovered a mechanism of ESX-5 regulation, but also identified a relevant environmental signal, phosphate limitation, that triggers ESX-5 activity (Elliott & Tischler 2016). Our lab previously demonstrated that phosphate-responsive gene regulation was mediated by the Pst/SenX3-RegX3 system in *M. tuberculosis* (Tischler et al. 2013). The Pst (phosphate specific transport) system transports inorganic phosphate across the inner membrane, and is induced when extracellular phosphate is scarce (Vanzembergh et al. 2010). The Pst system also plays a role in gene regulation through interaction with a two-component signal transduction system (Lamarche et al. 2008). The relevant system in *M. tuberculosis* is SenX3-RegX3, a membrane bound sensor kinase and a DNA-binding response regulator, respectively (Tischler et al. 2013). SenX3-RegX3 is activated by phosphate limitation, and is inhibited by the Pst system when phosphate is abundant. Our work has established that deletion of *pstA1*, a transmembrane component of the Pst system, results in constitutive activation of RegX3, regardless of phosphate abundance (Tischler et al. 2013). Disruption of regulation mediated by Pst/SenX3-RegX3 at any level causes attenuation of *M. tuberculosis in vivo*, highlighting the importance of phosphate sensing for the survival of the bacterium (Parish et al. 2003; Tischler et al. 2013).

In our most recent work, we provide evidence that the phosphate responsive Pst/SenX3-RegX3 system directly regulates ESX-5 at the transcriptional level. We found that disruption of the Pst system, through deletion of *pstA1*, resulted in significant upregulation of *esx-5* transcripts, along with increased production of ESX-5 core components and hypersecretion of known ESX-5 substrates (Elliott & Tischler 2016). We established that the changes in *esx-5* gene expression and secretion system activity seen in the Δ*pstA1* mutant required RegX3. We further demonstrated that ESX-5 secretion is induced by phosphate limitation. We observed overexpression of *esx-5* transcripts and overproduction and hypersecretion of ESX-5 substrates when *M. tuberculosis* was grown in medium with limiting phosphate. The induction of ESX-5 activity during phosphate limitation also required RegX3 (Elliott & Tischler 2016). Using electrophoretic mobility shift assays (EMSA), we demonstrated that RegX3 binds to a segment of DNA within the *esx-5* locus, suggesting that RegX3 directly activates ESX-5 secretion at the transcriptional level in response to phosphate starvation (Elliott & Tischler 2016). Our work is the first to show a direct link between the Pst/SenX3-

RegX3 and ESX-5 systems. Further work will more fully define the RegX3 binding site and characterize the importance of ESX-5 regulation during infection.

Throughout our experiments investigating ESX-5 regulation, we also monitored secretion of the ESX-1 substrate EsxB as a control to assess the effect of our experimental conditions on other secretion systems. To our surprise, EsxB was also hypersecreted in response to phosphate limitation through a RegX3-independent mechanism (Elliott & Tischler 2016). We observed no significant increase in *esxB* transcript abundance under phosphate starvation, suggesting induction of EsxB secretion occurs post-transcriptionally (Elliott & Tischler 2016). Hypersecretion of EsxB during phosphate scarcity may occur at the level of secretion or release of the protein. In *M. marinum,* and possibly *M. tuberculosis*, EsxB is found at the cell surface, and can mediate its virulence functions from this location (Kennedy et al. 2014). EsxB is also readily detected in the culture filtrate *in vitro*, though it is unclear whether release of the protein from the cell surface is passive or active. Perhaps phosphate limitation is one signal that actively triggers release of EsxB from the cell membrane. Nevertheless, our results suggest an additional unknown phosphate sensing mechanism, independent of RegX3, that activates EsxB secretion or release when phosphate is limited, which adds another layer of complexity to the regulation of ESX-1 secretion.

Phosphate limitation is a relevant environmental signal likely encountered by many microbial pathogens during infection (Lamarche et al. 2008; Yadav et al. 2015). For *M. tuberculosis,* the ability to sense and respond to starvation for this nutrient is critical to the success of the organism (Parish et al. 2003; Tischler et al. 2013;). There is evidence that *M. tuberculosis* is faced with phosphate limitation *in vivo*. Deletion of the gene encoding the phosphate binding component of the Pst system, which is predicted to impair phosphate uptake, results in a severe replication defect *in vivo* (Peirs et al. 2005). Moreover, the entire operon encoding the Pst system is required for survival in macrophages (Rengarajan et al. 2005). Future work in our lab will seek to pinpoint when and where *M. tuberculosis* encounters phosphate starvation using macrophage and murine infection models.

Regulation of ESX-5 secretion in response to phosphate limitation may be a critical function of the Pst/SenX3-RegX3 system for *M. tuberculosis* virulence. Both Δ*regX3* and Δ*pstA1* mutants are attenuated *in vivo* (Parish et al. 2003; Tischler et al. 2013). Deletion of *regX3* may cause attenuation due to an inability to up-regulate ESX-5 secretion when the bacteria encounter phosphate-limiting conditions. Conversely, the Δ*pstA1* mutant, in which RegX3 is constitutively activated, could be attenuated due to inappropriate constitutive hyper-secretion of ESX-5 substrates, some of which are highly antigenic (Sayes et al. 2012). Precise regulation of ESX-5 secretion in response to environmental signals, including phosphate, may be essential for *M. tuberculosis* to evade the host adaptive immune response to these antigens.

As previously discussed, several components of the ESX-5 core complex are essential for viability of *M. tuberculosis in vitro*. However, the essentiality of ESX-5 can be reversed by increasing the permeability of the outer membrane, by either introducing a porin or altering the lipid profile (Ates et al. 2015). This suggests that ESX-5 is essential for the secretion of proteins involved in nutrient uptake. This is an intriguing possibility, given that the ESX-3

system also functions in nutrient acquisition. Since ESX-5 secretion is induced in response to phosphate limitation, ESX-5-secreted proteins may mediate uptake of nutrients containing phosphate. The ESX-1 secreted substrate EspB adopts a fold that is similar to a previously characterized PE and PPE protein pair, which leads to oligomerization and formation of a heptameric complex with a pore at the center (Solomonson et al. 2015). Perhaps some ESX-5 associated PE and PPE proteins form similar oligomeric complexes that enable nutrient acquisition during phosphate limitation by forming pores in the outer membrane.

In addition to its role in nutrient uptake, ESX-5 may also play a more direct role in *M. tuberculosis* pathogenesis. There is precedence for multiple independent functions mediated by one ESX system. The ESX-3 system secretes substrates that have separable functions in iron acquisition and virulence independent of iron (Tufariello et al. 2016). We speculate that some ESX-5 substrates are also involved in the virulence of *M. tuberculosis* by modulating the host response. An as yet unknown ESX-5 secreted substrate(s) manipulates infected macrophages to undergo necrotic cell death, a function that seems unlikely to be related to nutrient uptake (Abdallah et al. 2011). Perhaps phosphate limitation is a signal that *M. tuberculosis* encounters in a particular host environment, like a phagosome, and ESX-5 effector proteins are deployed to promote survival in that niche. Current evidence certainly leaves open the possibility that ESX-5 substrates have multiple independent functions.

Our work has identified phosphate starvation as a novel environmental signal that activates ESX-5 secretion, demonstrated that this signal is communicated by the Pst/SenX3-RegX3 system, and revealed that this signal also leads to hyper-secretion of the ESX-1 substrate EsxB. These discoveries have provided hints toward a potential function of ESX-5 in phosphate acquisition and suggest that phosphate starvation is a nutritional cue that *M. tuberculosis* encounters in the host. Further work will be required to tease apart the potential nutrient uptake and virulence functions of ESX-5 by identifying ESX-5 effector proteins involved in these processes and to establish when and where *M. tuberculosis* encounters environments with limited phosphate during infection. Additional studies will also be necessary to determine the mechanism regulating secretion or release of the ESX-1 substrate EsxB in response to phosphate availability. We expect that further investigation of ESX effector proteins and the regulation of their secretion will greatly enhance our understanding of the interplay between the host and pathogen, and perhaps reveal new therapeutic targets.

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