Disconnecting *In Vitro* ESX-1 Secretion from Mycobacterial Virulence

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In this issue of the *Journal of Bacteriology*, Chen and colleagues (1) report a novel observation regarding the mycobacterial ESX-1 (ESAT-6 system-1/type VII) protein secretion system. Although the mechanisms underlying their observations are not yet understood, their exciting results unlink the *in vitro* secretion of the two major ESX-1 substrates, ESAT-6 (EsxA) and CFP-10 (EsxB), from *M. tuberculosis* virulence for the first time. These findings force us to reconsider how we relate the read-out of *in vitro* secretion assays to ESX-1 function within an infected host and call into question the roles of ESAT-6 and CFP-10 during infection.

CURRENT UNDERSTANDING OF THE MYCOBACTERIAL ESX-1 SECRETION SYSTEM

The ESX-1 (type VII) secretion system is required for the virulence of several Gram-positive and mycobacterial pathogens, including *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Bacillus anthracis* (2–5). ESX-1 is also conserved and functional in nonpathogens, where it plays vital roles in cell conjugation and physiology (6–9). In pathogens, ESX-1 functions early during infection when *Mycobacterium* is within the phagosome of the macrophage (4, 5). Specifically, ESX-1 is required for lysis of the phagosomal membrane, which allows either mycobacterial products or the bacteria access to the macrophage cytoplasm (10–14). Once lysis occurs, the cytosolic STING/TBK1 pathway is triggered, both promoting a type I interferon response and restricting bacterial growth through autophagy (11, 14, 15). This was recently reviewed in reference 16.

At the molecular level, ESAT-6 and CFP-10 are widely acknowledged as the major substrates of the ESX-1 secretion system. ESAT-6 has been reported to have membrane-lysing activity and may play a direct role in disrupting the phagosomal membrane (17–20). The first papers linking individual genes to the secretion of ESAT-6 and CFP-10 were published about a decade ago (4, 5, 21, 22). The majority of the molecular biology underlying ESX-1 export has been elucidated by turning on the system *in vitro*, fractionating mycobacterial cells to isolate whole-cell lysates and culture supernatants, and detecting the export of ESX-1 substrates by Western blot analysis or proteomics. Using this *in vitro* secretion assay, genes located in three distinct chromosomal loci were implicated in the export of ESX-1 substrates into the culture supernatant during growth in culture. Genes required for ESX-1 export lead to the loss of ESX-1 substrate secretion, most often ESAT-6 and CFP-10, *in vitro*. Reported loss of ESX-1 function *in vitro* has corresponded with attenuation of virulence both *ex vivo* and *in vivo* (primary examples include references 4, 5, 21, and 22).

UNLINKING *IN VITRO* ESX-1 SECRETION FROM VIRULENCE: A CHANGE IN PARADIGM

Chen et al. report for the first time a scenario where ESX-1 secretion appears to be disrupted *in vitro* but does not result in attenuation of *M. tuberculosis* in various infection models (1). The au-

thors focused on EspA, a known ESX-1 substrate. EspA, ESAT-6, and CFP-10 mutually require each other for export (23, 24). Several of the known ESX-1 substrates contain a WXG motif (25). The authors used site-directed mutagenesis to determine if the WXG motif in EspA was required for the secretion of CFP-10 and ESAT-6 from and virulence of *M. tuberculosis*. The expected outcome of this experiment was that mutations in EspA that prevented the export of ESAT-6 and CFP-10 would also lead to attenuation of *M. tuberculosis* in both cellular models of infection and in mice. Indeed, the majority of EspA variants generated in this study that prevented export of ESAT-6 and CFP-10 into the culture supernatant were noncytotoxic to macrophages, failed to induce proinflammatory cytokines, and were attenuated during acute infection in a mouse model. In short, they exhibited the expected phenotypes associated with ESX-1 deficiency.

Two EspA variants (EspAF5R and EspAK41A) resulted in *in vitro* protein secretion phenotypes that were identical to that of an *espA*-null strain (ESX-1 deficient); ESAT-6 and CFP-10 were produced but not exported into the culture supernatant. The EspAF5R and EspAK41A proteins were not detectable by Western blot analysis, indicating that these individual mutations destabilized the EspA proteins. However, although ESX-1 deficient *in vitro*, the strains expressing EspAF5R and EspAK41 were cytotoxic to macrophages, induced proinflammatory cytokines, and were as virulent as the wild-type strain in a mouse model. Thus, for the first time, the authors report that despite an apparent loss of ESX-1 secretion *in vitro*, the *M. tuberculosis* strains were not attenuated for virulence.

ASSAYS FOR MEASURING ESX-1 SECRETION AND FUNCTION

The report by Chen et al. (1) calls into question whether the *in vitro* secretion assay is a reliable indicator of ESX-1 function within a host. There are two commonly used ways to induce ESX-1 secretion *in vitro*. First, ESX-1 secretion can be induced by growing the mycobacteria in Sauton's medium, a general defined broth for bacterial culture. *Mycobacterium smegmatis* is a nonpathogenic mycobacterial species that serves as a model for ESX-1 export in *M. tuberculosis*. In *M. smegmatis*, ESX-1 secretion is induced by growth in Sauton's broth *in vitro* but not by growth in Middlebrook 7H9 broth, a defined medium for mycobacterial growth (9). We have adapted this approach to induce ESX-1 on solid Sauton's agar to detect ESX-1 export (26). Thus far, the means of induction by growth in Sauton's medium or on agar

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remains unknown. Second, ESX-1 secretion is induced by contact with red blood cells (RBCs). *Mycobacterium marinum* is a pathogenic mycobacterial species that also serves as an established model for ESX-1 secretion. *M. marinum* lyses RBCs in a contact-dependent, ESX-1-dependent manner (27, 28). RBC lysis is used to determine if the membrane lysing activity of the ESX-1 system is functional. The proteins secreted by ESX-1 following each type of induction have not been compared. Therefore, we do not know if both means of induction are equivalent with respect to the secreted proteome. Additional assays have been proposed to identify secreted substrates of ESX-1, but these have generally failed because tagging ESX-1 substrates with large, enzymatically active proteins generally prevents export (29).

The ESX-1 secretion system causes cytotoxicity in cell-based *ex vivo* models of infection, including macrophages and amoebae (12, 27, 30). Following lysis of the phagosomal membrane, the bacteria induce cytotoxicity, which leads to cell death. Cell death or perforation of the cell membrane is detected by the uptake of membrane-impermeable dyes or live/dead dyes. While the ESX-1 system functions in the phagosome, the proteins actively secreted by ESX-1 within the host cell remain elusive. We as a field have been unsuccessful at visualizing active secretion or localizing ESX-1 substrates in a host cell. However, a recent study reported the presence of ESAT-6 in lysates from THP-1 cells when infected with ESX-1-proficient *M. tuberculosis* strains (31). In contrast, the active substrate secretion and substrate localization by alternate secretion systems (types III and IV) have been directly visualized in host cells (recently reviewed in reference 32).

EVOLVING MODELS FOR ESX-1 EXPORT OF ESAT-6 AND CFP-10

Considering the long-standing correlation between *in vitro* secretion and ESX-1 mediated virulence, how do we explain the novel findings of Chen et al.? How can a mycobacterial cell that fails to secrete ESAT-6 and CFP-10 *in vitro* still be virulent *in vivo*? Although the studies by Chen et al. do not yet address mechanism, several potential models can be postulated.

As suggested by Chen et al. (1), the disconnect between *in vitro* ESX-1 secretion phenotypes and virulence could simply be explained by the limits of protein detection by Western blot analysis. The EspA variants (EspAF5R and EspAK41A) may be produced at "sub-Western" levels. Because EspA is required for ESAT-6/CFP-10 export, these variants may lead to "sub-Western" ESAT-6 and CFP-10 secretion. Low levels of ESAT-6 and CFP-10 may be sufficient to mediate virulence in a host cell. Alternatively, Chen et al. suggest that the instability of the EspAF5R and EspAK41A proteins *in vitro* may be stabilized in a host cell, allowing increased ESX-1 secretion *in vivo* (1).

Either explanation raises the question of whether the levels of ESAT-6 and CFP-10 secreted *in vitro* are reflective of the levels secreted in cell-based or animal infection models. For example, ESAT-6 and CFP-10 make up the majority of the secreted mycobacterial proteome *in vitro* and can be visualized by Coomassie staining of acrylamide gels. However, the active secretion of ESAT-6 and CFP-10 by ESX-1 has not yet been routinely visualized in infection models. Mechanistically, one could imagine that ESAT-6 and CFP-10 are secreted from the bacteria until they or another ESX-1 secreted protein interacts with the phagosomal membrane. Interestingly, Chen and colleagues recently published a report which showed that the EspB substrate directly interacts

with phospholipids (33). Interaction of a secreted ESX-1 component or substrate (possibly EspB) with the host membrane could signal to the ESX-1 system to switch or control the repertoire of secreted substrates in a model similar to those worked out for type III secretion systems (reviewed in references 34, 35, and 36). *In vitro*, the signal to stop ESAT-6/CFP-10 secretion may be missing (i.e., host membrane), allowing uncontrolled secretion of ESAT-6 and CFP-10 to high levels at the cost of export of additional ESX-1 substrates.

Although less likely, the strains expressing EspAF5R or EspAK41A may not secrete ESAT-6 and CFP-10 *in vitro* or in infection models. In this case, the *in vitro* assay accurately reflects the state of the ESX-1 system. Interaction with the host cell or a host protein may compensate for a loss of ESAT-6/CFP-10 secretion in infection models. Alternatively, mycobacterial factors could compensate for the loss of ESAT-6 and CFP-10. For example, although EspAF5R and EspAK41A expression led to secretion profiles similar to those of EspA-null strains as determined by Western blotting for the proteins measured by Chen et al. (1), there may be other changes to the secreted proteome not detected in this report. There are additional known *M. tuberculosis* ESX-1 substrates (EspE and EspC) that were not monitored in this study. What are the secretion phenotypes of the other substrates in the EspAF5R- and EspAK41A-expressing strains compared to the EspA null strain *in vitro*? What if these proteins (or others yet undiscovered) are secreted to higher levels when EspAF5R or EspAK41A is expressed, and these proteins along with ESAT-6 and CFP-10 mediate virulence? Are there other mycobacterial factors expressed and secreted in the host that can compensate for the loss of ESAT-1 and CFP-10 secretion? Again, because we are blind to the proteins that actually make up the ESX-1 complex and the secreted proteome in the host cell, we cannot exclude this possibility.

Another formal possibility is that expression of EspAF5R or EspAK41A proteins could bypass the requirement for ESAT-6 and CFP-10, allowing virulence in their absence. This type of bypass could occur if ESAT-6 and CFP-10 are secreted parts of the ESX-1 apparatus (37). Indeed, we observed that altering the EspC substrate could bypass the need for EspA in ESX-1 secretion *in vitro* (38).

The studies by Chen et al. draw attention to the need for continued development of assays for studying type VII secretion. More sensitive assays for detecting secretion are required, as the current assays may not be sensitive enough to detect *in vivo* levels of ESX-1 secretion. Clearly, the research reported here by Chen et al. (1) illustrates the fact that we do not understand all of the players in ESX-1 export, either on the mycobacterial side or on the host side. Therefore, more studies are required to identify bacterial and host genes required for ESX-1 export both *in vitro* and in cell-based models of infection. Finally, this research opens the door to exciting new ways to think about ESX-1 secretion, which will help direct us toward a better understanding of the interplay between mycobacterial pathogens and their hosts.

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