Mycobacterial virulence and specialized secretion: same story, different ending

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The genomic region lost during the attenuation of BCG vaccine encodes a newly discovered secretion system conserved among gram-positive bacteria. A series of papers has now dissected the components of this system, revealing a unique *Mycobacterium tuberculosis*–specific signal for export of bacterial proteins into the host.

Since its introduction as a public health intervention in 1921, vaccination with Bacillus Calmette-Guérin (BCG) has a long and controversial history. As a tool to study the pathogenesis of tuberculosis, BCG has a much shorter, but no less interesting, history. Despite its excellent safety profile, how BCG originally lost its virulence was unclear for decades, until Mahairas and colleagues performed a conceptually simple experiment[1]. Using subtractive hybridization to look for genomic regions absent from the attenuated *M. bovis* BCG vaccine strains, these investigators uncovered a nine-gene region of difference—RD1—as a candidate for the attenuation of BCG.

The potential importance of RD1 was reinforced when more comprehensive genomic comparisons did not find a more compelling candidate for the attenuation of BCG[2], and two naturally attenuated members of the *M. tuberculosis* complex—the Vole bacillus and the Dassie bacillus—were also found to have deletions in the RD1 region[3,4]. Complementation and gene disruption studies then established that RD1-encoded genes were required for the full virulence of *M. tuberculosis*[5–7]. We now had an explanation for the attenuation of BCG, but what was the mechanism? In a series of papers dissecting RD1 locus genes and their function, Jeffrey Cox and colleagues have made significant headway on this question[8–10].

An important clue was that two of the genes within RD1—*esat-6* and *cfp-10*—encoded extracellular proteins—ESAT-6 and CFP-10—useful in the immunodiagnosis of latent tuberculosis infection. Cox and others showed that disruption of individual RD1-region genes did not prevent production of ESAT-6 or CFP-10. However, an intact RD1 region was required to ensure that these proteins were secreted by the bacterium[8,11,12]. This virulence region was therefore identified as a new specialized secretion system with an unknown purpose.

Specialized secretion systems are hallmarks of virulence in Gram-negative bacteria, but Gram-positive organisms, which lack an outer membrane, were thought not to require such elaborate secretion machinery. However, the mycobacterial cell wall is a formidable hydrophobic barrier, and secreted molecules may need extra help to pass through it. In fact, this requirement may be much more common among Gram-positive bacteria than previously thought. Genomics and bioinformatics show that genes homologous to those in RD1 are conserved among pathogenic and nonpathogenic mycobacteria (**Table 1**), and more distant homologs to RD1 are widespread among other Gram-positive bacteria[13].

So, what is the function of this secretion system of *M. tuberculosis*? In addition to providing genetic evidence that RD1 encodes a secretion machinery, Cox's group also defined a role for this system in the dialog between the bacterium and host cell. First, they showed[8] that proteins encoded by *Rv3870* and *Rv3871* interact with ESAT-6 and CFP-10, and that deletion mutants of *Rv3870* and *Rv3871* fail to secrete those two molecules. *In vivo* studies in mice showed that individual mutants of *Rv3870, Rv3871* and *Rv3877* had decreased virulence similar to that of the *esat-6* mutant, and *ex vivo* studies in bone marrow–derived macrophages pointed to increased signaling by macrophages infected with the mutants. As macrophages infected with *M. tuberculosis* secrete less tumor necrosis factor-α and interleukin-12 than those infected with nonpathogenic mycobacteria, their finding suggested that RD1 might provide *M. tuberculosis* a means to actively alter host responses.

More recent findings have extended the boundaries of this system to another locus in the *M. tuberculosis* genome. It had been previously noted that genes *Rv3614c–Rv3616c* and genes immediately upstream of the RD1 locus shared some similarity, and that disruption of these genes resulted in severe attenuation of infection *in vivo*[14]. Cox and colleagues showed that the Δ*Rv3615c* strain produced (but did not secrete) ESAT-6 and CFP-10, and showed that Rv3614c can physically interact with Rv3882, a protein from the extended RD1 locus[9]. In a parallel, independent study[15], Fortune and colleagues showed that a strain lacking Rv3616c was unable to secrete ESAT-6 and, conversely, that disruption of RD1 prevented secretion of Rv3616c. The system was now gaining in complexity.

In a related contribution, Champion and colleagues revealed how a small genetic alteration may provide opportunities to re-tune this well conserved system. Using yeast two-hybrid analysis and gene truncations, the authors set out to map protein interactions among members of the RD1 system[10]. They determined that the terminal seven residues of

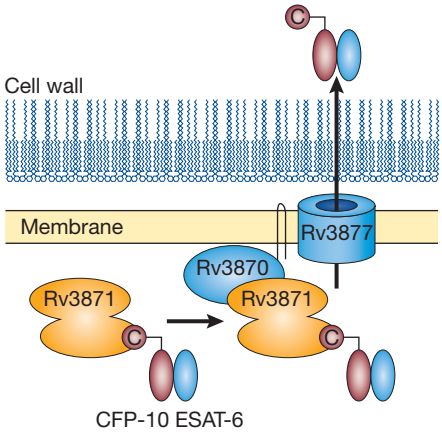

Figure 1 A model of the *M. tuberculosis* secretion system coded by the RD1 region. ESAT-6 and CFP-10 form a dimer in the cytoplasm before targeting. Rv3871 recognizes the C-terminal domain of CFP-10, targeting it together with ESAT-6 and CFP-10. Rv3871 then interacts with Rv3870, a membrane-bound component of the secretory system, linking the whole complex to the membrane. Modified from ref. 10.

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Table 1 Distribution of the RD1 region encoding a specialized secretion system across selected mycobacteria

Species	RD1 region	Notes
M. tuberculosis complex		
M. tuberculosis	Present	Primary cause of human tuberculosis
M. africanum	Present	Causes human tuberculosis in West Africa
Vole bacillus	Absent	Attenuated, used as human vaccine
Dassie bacillus	Absent	Attenuated virulence in animal models
M. bovis	Present	Primary cause of bovine tuberculosis
M. bovis BCG	Absent	Attenuated vaccine strain
Nontuberculous mycobacteria		
M. marinum	Present	Causes tuberculosis-like disease in fish
M. ulcerans	Absent	Cause of Buruli ulcer; unusual extracellular mycobacterial pathogen
M. leprae	Present	Cause of leprosy
M. kansasii	Present	Causes a tuberculosis-like pulmonary disease
M. avium complex	Absent	Complex of environmental and pathogenic mycobacterial species
M. smegmatis	Present	Rapidly growing, nonpathogenic mycobacterium

CFP-10 were sufficient for its interaction with Rv3871, and that single amino-acid substitutions within four of these seven amino acids blocked interaction (**Fig. 1**). Moreover, they directly implicated this motif in the targeting of proteins for export, by showing that expression of CFP-10 lacking these seven residues resulted in production of ESAT-6 and CFP-10, but not their secretion. To test the specificity of this targeting, they expressed ubiquitin fused to these seven amino acids in *M. tuberculosis* and showed that the addition of these residues was sufficient for secretion of this heterologous protein. Interestingly, alignment searches against paralogs in the *M. tuberculosis* genome and homologs in other species show that the terminal residues of the *M. tuberculosis* CFP-10 are unique within this protein family. These data therefore provide a potential explanation of how *M. tuberculosis* has evolved a novel use for a conserved secretory apparatus.

All of these findings raise further questions. Does RD1 facilitate secretion of other factors? Is ESAT-6, with or without CFP-10, the effector molecule that wreaks havoc on host cells[7], or do the key effectors still await description? Although it is clear that disruption of the system alters host response in potentially profound ways, what are the exact bacterial components responsible for the subversion of the host, and which specific host responses are necessary to keep mycobacteria in check? To what degree does the deletion of RD1 explain the attenuation of BCG, and did the absence of RD1 shape further *in vitro* evolution of BCG vaccine strains[16]? As there are paralogous regions to RD1 throughout the genome, are there specific signals unique to each of them? More generally, the evolution of this particular system provides a potential route by which organisms evolving toward a minimal genome, such as pathogenic mycobacteria, may fine-tune existing systems for novel purposes. How many other genes in the *M. tuberculosis* genome will be found to contain an extra few residues, permitting a subtle deviation in expression, signaling or function compared to their counterparts in other mycobacteria? In an era of increasingly rich genomic data, we are reminded that for bacteria, as for humans, when genetic similarity is high, it is the differences that are most interesting.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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