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MOLECULAR ASPECTS

Expression of OmpATb is dependent on small membrane proteins in *Mycobacterium bovis* BCG

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SUMMARY

Small membrane proteins emerge as a novel class of regulatory molecules in bacteria. Experiments carried out in *Mycobacterium bovis* BCG indicate that the *ompATb* gene (*Rv0899*), encoding a major outer membrane protein, is organized in operon with *Rv0900* and *Rv0901*, encoding two small proteins with a predicted transmembrane domain. Fractioning experiment confirmed the association of Rv0901 with the membrane fraction. To investigate the role of Rv0900 and Rv0901 in *M. bovis* BCG, we have constructed a strain deleted for the whole operon as well as complemented strains carrying a deletion of *Rv0900* or a frameshift mutation in either *Rv0900* or *Rv0901*. Importantly, mutations in *Rv0900* and/or *Rv0901* strongly altered OmpATb expression, demonstrating that Rv0900 and Rv0901 play a regulatory role, which appears to occur at a post-transcriptional level.

Mycobacteria possess an unusual cell envelope consisting of a cytoplasmic membrane and a highly impermeable lipid-rich cell wall.[1] Cryo-electron microscopy techniques recently allowed the visualization of an outer membrane in the mycobacterial cell envelope.[2–4] However, characterization of outer membrane proteins in mycobacteria remains a challenging task[5] and regulation of expression of these proteins is largely unknown. To date, the pore-forming protein OmpATb (Rv0899) represents one of the best characterized outer membrane proteins in *Mycobacterium tuberculosis*. The C-terminal domain of OmpATb displays weak similarity to the *Escherichia coli* OmpA protein.[6] However, recent structural data have indicated the presence of a mixed α/β structure and not a classical transmembrane β-barrel.[7,8] Recombinant OmpATb formed channels in lipid bilayers[6,9,10] and uptake of serine was found to be reduced in a mutant of *M. tuberculosis* lacking *ompATb*.[11] Phenotypic analysis of a mutant of *M. tuberculosis* lacking *ompATb* suggested that OmpATb is important for resistance to acid stress and virulence, being involved for intramacrophagic survival and replication of *M. tuberculosis* in infected mice.[11]

The *M. tuberculosis ompATb* gene is followed by two genes, *Rv0900* and *Rv0901*, encoding small predicted membrane proteins.[12] Since recent studies conducted in Gram-negative bacteria have highlighted the regulatory role of small membrane proteins,[13] we have investigated the relationship between OmpATb and Rv0900/Rv0901.

1. The *ompATb* gene is associated and co-transcribed with two small ORFs

In *M. tuberculosis*, the *ompATb* gene *(Rv0899)* is followed by *Rv0900*, which starts 12 bp after the *ompATb* stop codon and encodes a 50 amino-acid-long protein, and *Rv0901*, which has an ATG codon overlapping the *Rv0900* stop codon and encodes a 175 amino-acid-long protein (Figure 1A). Both Rv0900 and Rv0901 have a predicted transmembrane domain. Interestingly, both ORFs are conserved in mycobacterial species that harbor the *ompATb* gene (Figure 1B), with full conservation of the genetic organization, but absent in mycobacterial genomes that lack *ompATb* gene such as the slow-growing species *Mycobacterium avium* and the fast-growing mycobacteria *Mycobacterium smegmatis* and *Mycobacterium abscessus* (Figure 1B). To investigate whether these three

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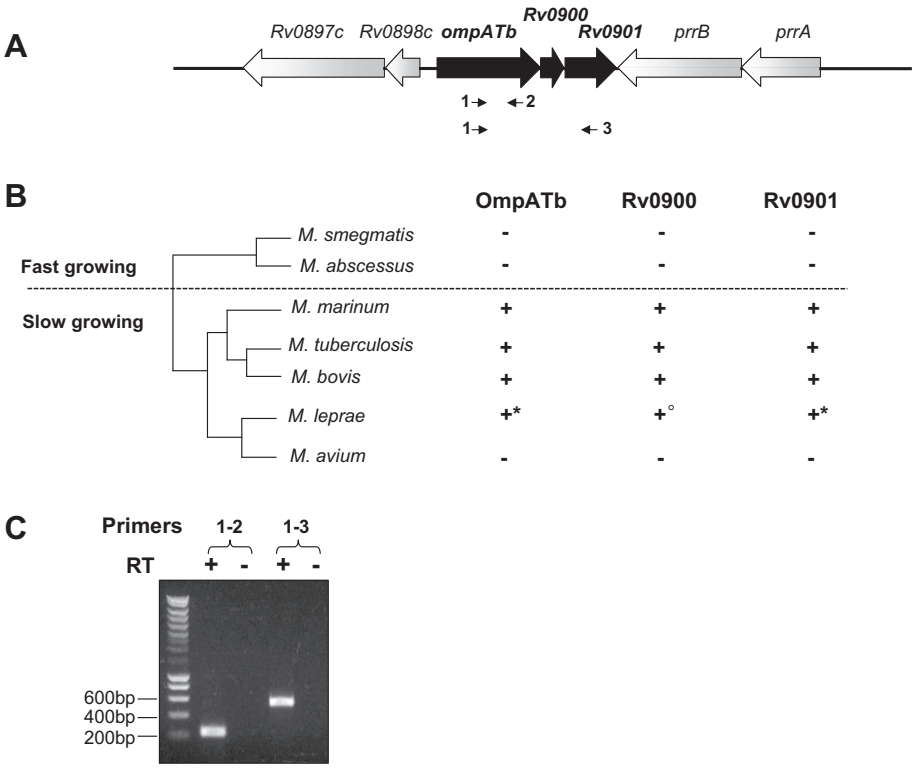

Figure 1. *The ompATb gene is co-transcribed with Rv0900 and Rv0901.* (A) Genomic organization of the *ompATb* locus in *M. tuberculosis* H37Rv. The *ompATb* gene is followed by two small ORFs, *Rv0900* and *Rv0901*. (B) Distribution of OmpATb, Rv0900 and Rv0901 proteins among mycobacterial species based on blastp and tblastn analysis (NCBI). A schematic representation of the phylogenetic tree showing the relatedness of the mycobacteria is shown. In *M. leprae*, pseudogenes (indicated by an asterix) corresponding to *ompATb* and *Rv0901* are found at the same location on the genome. Between these two pseudogenes, a non-annotated ORF (indicated by °) encoding a 50 amino-acid-long protein with homology to Rv0900 is found. (C) RT-PCR experiment on RNA isolated from wild-type *M. bovis* BCG. For DNA amplification from cDNA, denaturation was followed by 25 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s. Positions of internal primers to the *ompATb* gene (primers 1 and 2; to amplify a 261-bp fragment) and primers that map across *ompATb* and *Rv0901* (primers 1 and 3; to amplify a 586-bp fragment) are indicated in (A). Reverse transcriptase was omitted in two lanes (as indicated by −) to verify the absence of genomic DNA contamination in the RNA sample.

genes form a transcriptional unit, RT-PCR experiments were carried out on *Mycobacterium bovis* BCG RNA. The *M. tuberculosis* genes *Rv0899*, *Rv0900* and *Rv0901* being strictly identical to *M. bovis* BCG genes, *BCG_0951*, *BCG_0952* and *BCG_0953*, respectively, we will refer hereafter, for the sake of clarity, to the *M. tuberculosis* H37Rv gene nomenclature. RNA extraction and reverse transcription were performed as previously described.[14] DNA amplification was performed with GoldStar mix (Eurogentec) using primers within *ompATb* or primers overlapping *ompATb* and *Rv0901* (Figure 1A and Table S2). Amplification of a fragment extending from *ompATb* to *Rv0901* (Figure 1C) revealed a polycistronic mRNA including *Rv0900-Rv0901*. As expected, no fragment could be amplified using primers overlapping *Rv0901* and the adjacent gene *prrB* that is part of a divergent operon (data not shown). The strict association between *ompATb* and *Rv0900-Rv0901* in various genomes and their organization in an operon strongly suggests a functional link between these three ORFs.

2. Construction of *M. bovis* BCG strains mutated in *ompATb-Rv0900-Rv0901* operon and phenotypic analysis

A strain deleted for the whole operon *ompATb-Rv0900-Rv0901* (Δ*operon*) was created in *M. bovis* BCG using mycobacteriophage-mediated allelic exchange[15] to replace the operon by a hygromycin cassette (Figure 2A; Supplemental Material). The mutant was checked by Western blotting using OmpATb specific antibodies. To raise these antibodies, a recombinant truncated OmpATb protein ($OmpATb_{73-326}$) was purified essentially as described previously[9] and 100 μg of purified protein in phosphate-buffered saline mixed with Freund's incomplete adjuvant was injected subcutaneously three times in New-Zealand rabbits. Sera were collected and anti-$OmpATb_{73-326}$ antibodies further purified by protein A chromatography. Western blot analysis indicated that, as predicted, no more endogenous OmpATb was detected in the Δ*operon* BCG mutant (Figure 2B).

The BCG Δ*operon* mutant was then used for functional complementation studies to decipher a potential role of Rv0900 and Rv0901. This was done by introducing the integrative plasmid pMV306 (that integrates at the chromosomal *attB* locus) carrying either the entire *ompATb-Rv0900-Rv0901* locus, designated as pOperon, or the *ompATb* gene alone, designated as pOmpA (Figure 2A and Table S1). In addition, three plasmids derived from pOperon were constructed to obtain complemented strains that carry the *ompATb* gene but lack functional Rv0900 or Rv0901 (Figure 2A and Table S1). First, inverse PCR was carried out on pOperon to delete *Rv0900*, generating pOmpA-Rv0901. Then, site-directed mutagenesis was performed on pOperon to introduce a stop mutation at the seventh codon of *Rv0900* or *Rv0901*, generating pStopRv0900 and pStopRv0901, respectively.

Strains with a stop mutation in *Rv0900* or *Rv0901* exhibited the same growth kinetics as the wild-type strain in Sauton medium (Figure S2), indicating that *Rv0900* and *Rv0901* are dispensable for *in vitro* growth in *M. bovis* BCG, although *Rv0900* was suggested to be essential by Himar-1-based transposon mutagenesis in *M. tuberculosis* H37Rv.[16] A previous study reported that a *M. tuberculosis ompATb* deletion mutant had a growth defect at pH 5.5 in Dubos medium and had a lower replication rate in macrophages although no complementation experiments have been conducted in