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Mol Microbiol. Author manuscript; available in PMC 2015 September 01.

Published in final edited form as:
Mol Microbiol. 2014 September ; 93(5): 1057–1065. doi:10.1111/mmi.12718.

EspI regulates the ESX-1 secretion system in response to ATP levels in *Mycobacterium tuberculosis*

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Summary

The function of EspI, a 70-kDa protein in *Mycobacterium tuberculosis,* has remained unclear. Although EspI is encoded by a gene within the *esx-1* locus, in this study we clarify previous conflicting results and show that EspI is not essential for ESX-1-mediated secretion or virulence in *M. tuberculosis.* We also provide evidence that reduction of cellular ATP levels in wild-type *M. tuberculosis* using the drug bedaquiline completely blocks ESX-1-mediated secretion. Remarkably, *M. tuberculosis* lacking EspI fails to exhibit this phenotype. Furthermore, mutagenesis of a highly conserved ATP-binding motif in EspI renders *M. tuberculosis* incapable of shutting down ESX-1-mediated secretion during ATP depletion. Collectively these results show that *M. tuberculosis* EspI negatively regulates the ESX-1 secretion system in response to low cellular ATP levels and this function requires the ATP-binding motif. In light of our results the potential significance of EspI in ESX-1 function during latent tuberculosis infection and reactivation is also discussed.

Keywords

Tuberculosis; ESX-1; secretion; EspI; ATP-binding motif

Introduction

The type-7 ESX-1 protein secretion system of *Mycobacterium tuberculosis* is encoded by 16 genes in the *esx-1* locus and the unlinked *espACD* locus (Bitter *et al.*, 2009). The majority of these genes are highly conserved in members of the *M. tuberculosis* complex as well as in other pathogenic mycobacteria such as *Mycobacterium leprae*, the agent of leprosy, and *Mycobacterium marinum*, a pathogen of ectothermic vertebrates (Bentley *et al.*, 2012, Cole

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et al., 1998, Cole *et al.*, 2001, Garnier *et al.*, 2003, Stinear *et al.*, 2008). This system is essential for the secretion of EsxA (ESAT-6), EsxB (CFP-10), EspA, EspB and EspC proteins, and plays a decisive role in the virulence of *M. tuberculosis* (Bitter *et al.*, 2009, Simeone *et al.*, 2009). Deletion of the entire *esx-1* locus or of genes contained therein abrogates ESX-1-mediated secretion and strongly attenuates *M. tuberculosis* (Brodin *et al.*, 2006, Guinn *et al.*, 2004, Hsu *et al.*, 2003, Lewis *et al.*, 2003, Pym *et al.*, 2002, Stanley *et al.*, 2003).

EspI is a 70-kDa alanine- and proline-rich protein of unknown function and it is encoded by the *espI* gene (*rv3876*) located in the *esx-1* locus. Bioinformatic analysis of the EspI amino acid sequence revealed an FlhG domain (Das *et al.*, 2011). The FlhG protein is needed to negatively regulate the number of polar flagella in *Vibrio cholerae*, as such it was proposed that EspI might play a role in regulating the ESX-1 system (Das *et al.*, 2011). An *espI* transposon insertion mutant of *M. tuberculosis* H37Rv was found to be defective in ESX-1 mediated secretion and attenuated (Guinn *et al.*, 2004). Complementation of this mutant with *espI* alone reportedly restored the wild-type phenotype (Guinn *et al.*, 2004). However, in a second study, an in-frame deletion mutant of *espI* was found to be as secretion-competent and virulent as the wild-type control strain suggesting that EspI is not an essential component of the ESX-1 secretion apparatus (Brodin *et al.*, 2006). In light of these contradictory findings, the role of EspI in ESX-1-mediated secretion and virulence has remained puzzling and unclear.

In this study, we document a novel role of EspI in ESX-1 function. While not critical for ESX-1-mediated secretion or cytotoxicity, we provide evidence that EspI is involved in repressing ESX-1 activity under conditions of lowered cellular ATP levels in *M. tuberculosis*. We have also defined a conserved ATP-binding motif in EspI to be pivotal for this role.

Results

M. tuberculosis EspI is not essential for ESX-1-mediated secretion or cytotoxicity

The *espI* (*rv3876*) gene is 2001 bp in length and located immediately upstream of *eccD*$_1$ (*rv3877*) in the *esx-1* locus (Fig. 1A). In this study, an *M. tuberculosis* Erdman mutant with a transposon insertion at nucleotide position 1039 of the *espI* gene was used for detailed analysis (Fig. 1A). The *in vitro* growth rate of the *espI*::Tn mutant strain was found to be indistinguishable from that of wild-type *M. tuberculosis* (data not shown). Unlike wild-type *M. tuberculosis* however, the secretion of EsxA, EsxB and the mature form of EspB was deficient in the *espI*::Tn mutant strain (Fig. 1B). By infecting and measuring its capacity to kill THP-1 macrophage cells, the *espI*::Tn strain was also found to be less cytotoxic than wild-type *M. tuberculosis* but as attenuated as the 5′ Tn::*pe35* strain (Fig. 1C), shown previously to be deficient in ESX-1-mediated secretion and virulence (Chen *et al.*, 2013). These results indicate that the *espI*::Tn mutant strain has a non-functional ESX-1 secretion system and is attenuated in macrophage cytotoxicity.

As *espI* is located directly upstream of *eccD*$_1$, we examined if the transposon insertion in *espI* might be causing a polar effect on the transcription of *eccD*$_1$, which is essential for

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ESX-1 secretion (Brodin *et al.*, 2006, Pym *et al.*, 2002). To this end, reverse transcriptase (RT)-PCR analysis of total RNA isolated from wild-type *M. tuberculosis* was performed. An RTPCR product spanning the intergenic region of *espI* and *eccD*$_1$ was detected suggesting that they are co-transcribed (Fig. 2A). To further corroborate these results, *eccD*$_1$ transcript levels in wild-type and *espI*::Tn strains were measured by quantitative real time-PCR. The mutant strain produced almost 100-fold less *eccD*$_1$ mRNA compared to the wild-type, while *esxA* mRNA levels remained similar (Fig. 2B). These results show transposon insertion in the *espI* gene affects the transcription of *eccD*$_1$ and likely accounts for the lack of ESX-1-mediated secretion and THP-1 cytotoxicity observed for the *espI*::Tn mutant strain.

To verify that the lack of ESX-1 function in the *espI*::Tn mutant strain resulted from polar effects on *eccD*$_1$ transcription, three different plasmids bearing *espI* or *eccD*$_1$ only or both genes - pMD*espI*, pMD*eccD*$_1$ and pMD*espI-eccD*$_1$, all under the control of the *espI* promoter, were constructed. After transformation into the *espI*::Tn strain, levels of *espI* and *eccD*$_1$ transcripts were measured using quantitative real time-PCR. All three transformants were found to produce *espI* and/or *eccD*$_1$ mRNA effectively according to the plasmid constructs they harboured (Fig. S1). ESX-1-mediated secretion of these strains was then analyzed. Equivalent amounts of EsxA protein were detected in the culture filtrates of wild-type *M. tuberculosis*, *espI*::Tn strains transformed with pMD*espI-eccD*$_1$ and pMD*eccD*$_1$, but not pMD*espI* or empty pMD31 vector (Fig. 2C). The cytotoxicity of these strains was also measured using the THP-1 macrophage infection system described above. In contrast to *espI*::Tn strains with and without pMD31, *espI*::Tn transformed with pMD*espI-eccD*$_1$ and pMD*eccD*$_1$ were as cytotoxic to THP-1 macrophages as wild-type *M. tuberculosis* (Fig. 2D). However, the *espI*::Tn strain transformed with pMD*espI* remained attenuated (Fig. 2D). These results confirm *espI* and *eccD*$_1$ are co-transcribed and indicate that provision of *espI* and *eccD*$_1$ or *eccD*$_1$ but not *espI* alone to the *espI*::Tn mutant strain restores ESX-1-mediated secretion and THP-1 cytotoxicity.

ESX-1-mediated secretion is blocked when cellular ATP levels in *M. tuberculosis* is reduced

Three ESX-1 core component proteins, namely EccA$_1$, EccCa$_1$ and EccCb$_1$, contain putative ATPase domains and have been proposed to power the multimerization and translocation of ESX-1 substrates (Stoop *et al.*, 2012, Das *et al.*, 2011). We sought to address whether perturbations in ATP synthesis and lowered cellular ATP levels might impact ESX-1 function. For this purpose, we assessed the effect of treatment with bedaquiline (BDQ), a drug that specifically targets the *c* subunit of mycobacterial ATP synthase and inhibits ATP synthesis, on ESX-1-mediated secretion by wild-type *M. tuberculosis* (Andries *et al.*, 2005, Koul *et al.*, 2007). BDQ treatment for 8 hours at 0.015, 0.03 and 0.06 μg/ml decreased cellular ATP levels in *M. tuberculosis* by 0%, 43% and 71%, respectively, relative to the untreated control (Fig. S2). Treatment of *M. tuberculosis* with BDQ at 0.03 μg/ml, which is half the MIC (Andries *et al.*, 2005), completely blocked the secretion of EsxA, EsxB and EspB, while Ag85B secretion was partially affected compared to that of untreated cells (Fig. 3A). The effect of two cell-wall targeting antimycobacterial compounds, ethambutol (EMB) and isoniazid (INH) at 5 and 0.25 μg/ml respectively, on ESX-1-mediated secretion were also tested. EMB and INH at these concentrations had no effect on EsxA secretion despite

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being able to inhibit growth of wild-type *M. tuberculosis* to the same extent as BDQ at 0.03 μg/ml *in vitro* (Fig. S3). This suggests that blockage of ESX-1-mediated secretion by BDQ at 0.03 μg/ml is not due to the impaired growth of bacterial cells. Thus ESX-1 secretion is shut down when the ATP level in *M. tuberculosis* is reduced.

EspI is involved in blocking ESX-1 secretion during lowered cellular ATP levels in *M. tuberculosis*

Since EspI was predicted to negatively regulate ESX-1 function (Das *et al.*, 2011), we reasoned that it might play a role in the blockage of ESX-1-mediated secretion under conditions of low cellular ATP. To test this, the impact of BDQ on ESX-1-mediated secretion in *espI*::Tn strains harbouring pMD*espI-eccD*$_1$ and pMD*eccD*$_1$ was assessed. When treated with BDQ at 0.03 μg/ml, the *espI*::Tn mutant strain transformed with pMD*espI-eccD*$_1$ stopped secreting EsxA, EsxB and EspB much like wild-type *M. tuberculosis* (Fig. 3B). In stark contrast, the *espI*::Tn strain transformed with pMD*eccD*$_1$ continued to secrete EsxA, EsxB and EspB, even in the presence of BDQ (Fig. 3C). These results indicate that EspI is involved in the repression of ESX-1-mediated secretion under conditions of low cellular ATP in *M. tuberculosis*.

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The ATP-binding motif of EspI is required for blocking ESX-1 secretion during lowered cellular ATP levels in *M. tuberculosis*

EspI was shown to contain an FlhG domain (Das *et al.*, 2011). FlhG and FleN (FlhG-homolog) proteins in *Vibrio cholerae* and *Pseudomonas aeruginosa* respectively, regulate the number of flagella synthesized at the bacterial cell poles (Dasgupta & Ramphal, 2001, Correa *et al.*, 2005). Furthermore, a lysine residue in the conserved ATP-binding motif of *P. aeruginosa* FleN was shown to be crucial for its function (Dasgupta & Ramphal, 2001). Amino acid sequence analysis revealed that like FlhG and FleN, mycobacterial EspI proteins including those of *M. tuberculosis*, *M. leprae* and *M. marinum* contain a highly conserved ATP-binding motif (Fig. 4A). To determine if the ATP-binding motif might be crucial for EspI involvement in blocking ESX-1-mediated secretion, a conserved lysine residue 426 (K426) in this motif was replaced with glutamine by site-directed mutagenesis of pMD*espI-eccD1*, thus generating pMD*espI*^{K426Q}*-eccD1* (Fig. 4A). An *espI*::Tn strain transformed with pMD*espI*^{K426Q}*-eccD*$_1$ was then treated with BDQ at 0.03 μg/ml. In the absence of the drug, the EspI^{K426Q}-expressing strain (*espI*::Tn+ pMD*espI*^{K426Q}*-eccD*$_1$), like the fully complemented EspI^{WT}-expressing strain (*espI*::Tn+pMD*espI-eccD1*), secreted ESX-1 substrates normally. In the presence of BDQ however, the EspI^{K426Q}-expressing strain unlike the EspI^{WT}-expressing strain did not block ESX-1-mediated secretion and abundant amounts of EsxA, EsxB and EspB were found in the culture filtrate (Fig. 4B). The cytotoxicity of the EspI^{K426Q}-expressing strain was also assessed and it was found to kill THP-1 macrophages to the same extent as the wild-type and fully complemented strains (Fig. 4C). These results confirm that EspI is required to repress ESX-1-mediated secretion under low ATP conditions and show that its ATP-binding site is essential for this function.

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Discussion

Taking a genetic approach, we have confirmed in this study that EspI is not essential for the *M. tuberculosis* ESX-1 secretion system. However, we have discovered that EspI plays a novel role in negatively regulating ESX-1-mediated secretion under conditions where cellular ATP levels are depleted. Furthermore, we show that a conserved ATP-binding motif in the protein is required for this function.

Our data indicate that insertion of a transposon in *espI* causes a polar effect on the transcription of the downstream $eccD_1$ gene; consequently, the *espI*::Tn mutant strain should be regarded as a double knock-out of *espI* and $eccD_1$. The results of our complementation experiments confirm that EspI is not required for ESX-1-mediated secretion or THP-1 cytotoxicity and are consistent with the findings of Brodin and colleagues who had shown that an in-frame deletion of *espI* had no effect of EsxA secretion or on virulence (Brodin *et al.*, 2006).

Little is known regarding the impact of ATP availability on ESX-1-mediated secretion and virulence. Given that $EccA_1$, $EccCa_1$ and $EccCb_1$ contain putative ATPase domains and have been proposed to power ESX-1-mediated secretion (Stoop *et al.*, 2012, Das *et al.*, 2011) we surmised that an ATP deficit in *M. tuberculosis* might impact the secretion system. Indeed, treatment with sub-lethal concentrations of BDQ, a potent inhibitor of mycobacterial ATP synthesis, and the consequent reduction of cellular ATP levels in *M. tuberculosis* completely blocked ESX-1-mediated secretion *in vitro*.

EspI was suggested to negatively regulate ESX-1 function because it contains an FlhG domain (Das *et al.*, 2011). This stems from the fact that FlhG and FleN (FlhG homolog) proteins are required by *V. cholerae* and *P. aeruginosa,* respectively, to regulate the number of flagella formed at the poles (Dasgupta & Ramphal, 2001, Correa *et al.*, 2005). FleN-null and FlhG-null mutants of *P. aeruginosa* and *V. cholerae* respectively are hyper-flagellated. Conversely, cells overexpressing FleN and FlhG lack flagella (Dasgupta & Ramphal, 2001, Correa *et al.*, 2005). Polar flagellar assembly in *P. aeruginosa* and *V. cholerae* is a tightly regulated, spatio-temporally coordinated process of multi-protein synthesis and translocation of proteins from the cytoplasm to outside the bacterial cell (Kazmierczak & Hendrixson, 2013). It has become evident that FlhG (and FleN) through a variety of different mechanisms play pivotal roles in this complex process (Kazmierczak & Hendrixson, 2013).

Although the flagellar assembly machinery and the ESX-1 secretion system may appear to share mechanistic similarities, closer analysis reveals that FlhG/FleN and EspI are quite different. For instance, *V. cholerae* FlhG is 291 amino acids in length while *M. tuberculosis* EspI comprises 666 amino acids. Furthermore, the two proteins only share 23.9% sequence identity and likely function differently. This is consistent with our observations that EspI deficiency does not result in the hyper-secretion of ESX-1 substrates (or affect ESX-1 secretion at all) when the tubercle bacillus is ATP-replete. Nevertheless, our intriguing demonstration that BDQ blocks ESX-1-mediated secretion prompted us to investigate whether EspI might be involved in this phenomenon and led us to serendipitously find that *M. tuberculosis* lacking EspI (*espI*::Tn+pMD$eccD_1$) is rendered incapable of shutting down

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ESX-1-mediated secretion in the presence of BDQ. In addition to *M. tuberculosis,* EspI orthologues are also present in other pathogenic mycobacteria with ESX-1 systems such as *M. bovis*, *M. africanum*, *M. canetti*, *M. leprae, M. kansasii* and *M. marinum*. In *M. smegmatis,* a nonpathogenic species which appears to possess a functional *esx-1* locus, the *espI* orthologue is 51% identical to the *M. tuberculosis* counterpart and is missing 577 bp encoding the N-terminal portion of *M. tuberculosis* EspI (Converse and Cox. JBac.2005vol. 187p.1238). Furthermore, a conserved ATP-binding motif located in the amino-terminus of *P. aeruginosa* FleN appears to be crucial for its function although the precise mechanism remains unknown (Dasgupta & Ramphal, 2001). A similar ATP-binding motif was identified at the carboxy-terminus of EspI from *M. tuberculosis* and mycobacteria described above. The high degree of conservation of this motif among mycobacterial EspI proteins suggested it was of functional importance. Indeed, mutagenesis of a conserved K426 residue in the ATP-binding motif rendered *M. tuberculosis* incapable of repressing ESX-1 mediated secretion upon BDQ treatment and the consequent reduction in cellular ATP levels.

The precise molecular mechanism underlying EspI function and the role of the ATP-binding motif remain unknown and warrant further investigation. We hypothesize that a decline in ATP concentration within the tubercle bacillus may be sensed through the ATP-binding motif and confers a specific molecular conformation to EspI. This change in conformation could in turn trigger the transcriptional repression of core *esx-1* genes required for ESX-1 secretion. Alternatively, reduced protein synthesis and/or destabilization of core ESX-1 components might be another pathway whereby EspI could exert its secretion-repressing effect. A change in EspI conformation could also enable the protein to physically obstruct ESX-1-mediated secretion at the *M. tuberculosis* cell membrane via protein-protein interactions. Indeed, three independent proteomic studies have found EspI to be particularly enriched in the cell membrane fraction of *M. tuberculosis* cells (Mawuenyega *et al.*, 2005, Malen *et al.*, 2010, de Souza *et al.*, 2011).

Studies thus far have largely implicated transcriptional regulators, such as PhoP (Walters *et al.*, 2006, Gonzalo-Asensio *et al.*, 2008, Frigui *et al.*, 2008), EspR (Blasco *et al.*, 2012, Raghavan *et al.*, 2008) and MprAB (Pang *et al.*, 2013) in orchestrating ESX-1 gene expression. Here, EspI is shown for the first time to exert an additional layer of control which is dependent on cellular ATP levels in *M. tuberculosis*. Our data may therefore provide clues with respect to ESX-1 function during the chronic phase and even during latent *M. tuberculosis* infection. It is widely accepted that within granulomas and possibly during latent infection, ATP synthesis in non-replicating and dormant *M. tuberculosis* is significantly reduced (Gengenbacher & Kaufmann, 2012, Rittershaus *et al.*, 2013). However, little is known about ESX-1 function during this phase of *M. tuberculosis* infection. Repression of ESX-1-mediated secretion involving EspI during this stage might be an important way not only to maintain appropriate ATP homeostasis but also to balance the virulence-promoting function of ESX-1 (Pym *et al.*, 2002) and the T-cell stimulating properties of ESX-1 substrates (Pym *et al.*, 2003). As such, the observation that EspI in *M. tuberculosis* is detected 90 days but not 30 days post-infection in guinea pigs raises the intriguing possibility that EspI may be more important during the chronic and later phases than in the acute phase of infection (Kruh *et al.*, 2010). While we did not observe any impact

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of EspI deficiency in *M. tuberculosis* on THP-1 cytotoxicity, the short time-frame of our *ex vivo* experiments precludes us from ruling out possible effects on virulence during long-term chronic infection. For instance, decreased virulence during long-term *in vivo* infection might occur if EspI-deficient *M. tuberculosis* were unable to shut down ESX-1-mediated secretion, avoid presentation of the major immunogenic ESX-1 substrates and, as a consequence, elicit a more robust host immune response against the bacillus. In this context, manipulation of EspI in recombinant BCG strains complemented with the *esx-1* locus could yield more efficacious TB vaccines.

In conclusion, this study has increased our understanding of the regulation of ESX-1-dependent secretion. Better mechanistic insight into how EspI functions might reveal new modes of combating *M. tuberculosis* infection. For instance, small-molecules constitutively activating the ESX-1 inhibitory function of EspI, even under ATP-replete conditions, would make ideal anti-virulence drugs. In this respect, BDQ appears to act indirectly as an anti-virulence drug by lowering ATP-levels and thus ablating ESX-1 activity. Since the latter is required for reactivation of latent TB, it is conceivable that the highly pronounced bactericidal effect of BDQ on non-replicating *M. tuberculosis* seen *in vivo* may stem in part from inhibition of the ESX-1 system (Zhang *et al.*, 2012)

Experimental Procedures

Enzymes and reagents

Restriction and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA, USA). High fidelity *Pfu* polymerase was purchased from Promega (Madison, WI, USA). Cosmid 2F9 (Pym *et al.*, 2002) containing the *M. tuberculosis* H37Rv *esx-1* genomic region was used as template for PCR cloning. All other chemicals and reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bacterial strains and growth conditions

M. tuberculosis Erdman was grown in 7H9 broth (supplemented with 0.2% glycerol, 10% ADC and 0.05% Tween-80) or on 7H11 agar (supplemented with 0.5% glycerol, 10% OADC). *M. tuberculosis* Erdman Tn5370 transposon insertion mutants *espI*::Tn and 5′ Tn::*pe35* were generated as described elsewhere (Dhar & McKinney, 2010) and grown in the presence of hygromycin (50 μg/ml). *Escherichia coli* TOP10^{TM} (Invitrogen, Carlsbad, CA, USA) used for routine cloning was grown on Luria-Bertani agar or broth. When needed, kanamycin was used at a final concentration of 25 μg/ml for *M. tuberculosis* and at 50 μg/ml for *E. coli*.

Quantitative RT-PCR

Total mRNA was extracted from exponentially growing *M. tuberculosis* cells using TRIzol (Invitrogen, Carlsbad, CA). Purified mRNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI) according to the manufacturer’s instructions. Treated mRNA was checked for complete removal of contaminating genomic DNA by PCR before proceeding to the reverse transcription step. cDNA from mRNA was generated using the Superscript III first-strand synthesis system (Invitrogen, Carlsbad, CA) and random hexamers. Three

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different primer pairs were used to amplify internal fragments of *espI* and *eccD*$_{1,}$ and the portions encompassing the coding sequence and intergenic regions between the two genes from the cDNA generated as described above. To measure transcript levels of *espI* and *eccD*$_{1,}$ cDNA corresponding to 10 ng of input RNA was used in each RT-PCR reaction using 200 nM each of *espI, eccD*$_{1,}$ *esxA* and *sigA* specific primer pairs and SYBR-Green master mix (Applied Biosystems). Relative mRNA levels were calculated using the Ct method, normalizing transcripts levels *to sigA* signals. Data are shown as percentage relative to wild-type *M. tuberculosis.* All primer sequences are available upon request.

Vector construction and complementation

The *espI-eccD*$_{1}$ gene cluster including 147 bp upstream of the *espI* start codon was PCR-amplified from *M. tuberculosis* H37Rv cosmid 2F9 (Pym *et al.*, 2002) with primers that added 5′ and 3′ *Sbf*I sites. The PCR product generated was digested, purified, and ligated to the mycobacterial-*E. coli* shuttle vector pMD31 (Donnelly-Wu *et al.*, 1993) to obtain the episomal plasmid pMD*espI-eccD*$_{1,}$ which confers kanamycin resistance. Site-directed mutagenesis of *espI* in pMD*espI-eccD*$_{1}$ for the expression of EspI^{K426Q} mutant was performed using the Quickchange Site-Directed Mutagenesis Kit (Agilent Technologies) and corresponding oligonucleotides with their antisense strands (primer sequence available upon request). All constructs generated in this study were verified by DNA sequencing. *M. tuberculosis espI*::Tn mutant was grown to mid-logarithmic phase prior to electroporation of the above mentioned plasmids following standard procedures (Wards & Collins, 1996). *M. tuberculosis espI*::Tn transformants harbouring pMD*espI-eccD*$_{1}$ or its derivatives were selected on 7H11 agar plates containing 25 μg/ml kanamycin.

THP-1 cell culture and infection

To evaluate the virulence of different *M. tuberculosis* mutants, infection of human THP-1 monocytic cells was utilized. Actively dividing THP-1 cells grown in complete RPMI medium (Gibco RPMI supplemented with glutamine and 10% FCS) were treated with 10 nM phorbol myristate acetate (PMA), seeded at the required densities in multi-well plates and allowed to differentiate into adherent phagocytic cells over 3 days. PMA-containing medium was replaced with fresh complete RPMI medium and the cells incubated overnight before infecting with *M. tuberculosis* at a multiplicity of infection of 5. Cytotoxicity induced by *M. tuberculosis* was determined by measuring the metabolism of surviving THP-1 cells after 3 days post-infection with PrestoBlue Cell Viability Reagent (Life Technologies). Relative cytotoxicity of mammalian cells infected with different *M. tuberculosis* strains was measured as a percentage of uninfected cells in each experiment.

Protein preparation for immunoblots

Culture filtrates and cell lysates were prepared and immunoblotted as described previously (Chen *et al.*, 2012). *M. tuberculosis* grown to an OD $_{600nm}$ of 0.5 - 0.6 in Sauton's medium containing 0.05% Tween-80 was centrifuged, washed once with PBS, resuspended in Sauton's medium without Tween-80 with addition of tested compounds if needed, and grown further at 37 °C with shaking for 4 or 5 days. Culture filtrate proteins were obtained after centrifugation of cultures, filtering of the supernatant through 0.2 micron filters and

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100-fold concentration with 5-kDa-molecular-weight-cutoff membranes (Sartorius Stedim Biotech GmbH, Goettingen, Germany). Cell lysates were prepared from cell pellets lysed by bead beating in cold PBS containing Roche protease inhibitor cocktail. Total protein concentrations were determined by the BCA assay (Pierce) with bovine serum albumin as the standard. Indicated amounts of culture filtrate and cell lysate proteins were resolved in NuPAGE 4 to 12% Bis-Tris gels (Invitrogen, Carlsbad, CA, U.S.A.), transferred to nitrocellulose membranes and blocked with TBS-Milk (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 5% non-fat milk powder). Membranes were usually incubated overnight with the required primary antibody in TNT-BSA (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% BSA fraction V) at 4°C and with the appropriate secondary antibody in TNT-BSA for 30 min at room temperature and developed using Lumi-Light Plus chemiluminescence reagent (Roche, Mannheim, Germany).

GroEL2 was used as a lysis control for culture filtrates. EsxA, EsxB and EspB were detected using anti-EsxA mouse monoclonal antibodies (HYB 76-8), anti-EsxB rabbit serum, and anti-EspB rat serum, respectively.

Statistical analysis

The significance in differences between experimental groups was determined using Student's *t*-test (two-tailed, unpaired with equal variances) in the GraphPad Prism version 6 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Koen Andries for kindly providing BDQ and Ida Rosenkrands for anti-EspB rat serum. J.M.C. received post-doctoral fellowships from the Canadian Thoracic Society and the Canadian Institutes of Health Research. J.R. was supported by the German Federal Ministry of Research and Education (BMBF grant 01KI1017). This study received funding from the Swiss National Science Foundation (31003A-140778) and the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreements n°201762 and 260872. Antibodies against GroEL2 and Antigen-85 complex were received as part of the National Institutes of Health, National Institute of Allergy and Infectious Diseases contract (no. HHSN266200400091c) entitled "Tuberculosis Vaccine Testing and Research Materials", awarded to Colorado State University. The authors have no conflicts of interest.

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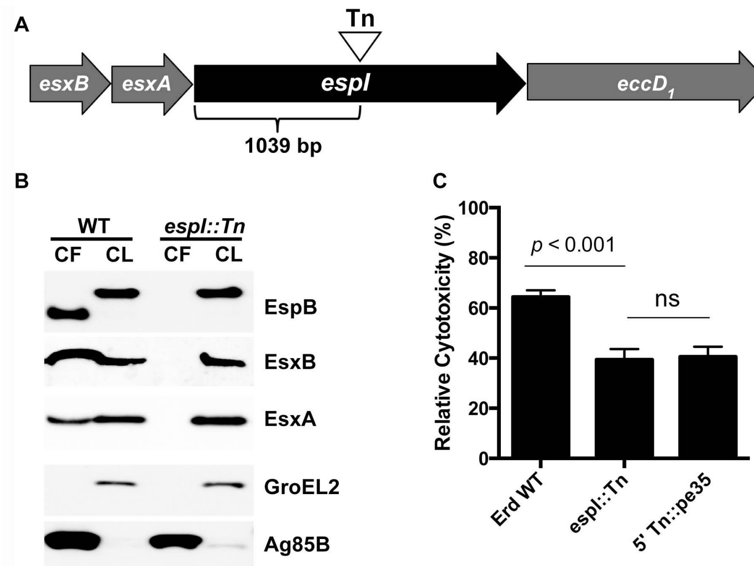

Fig. 1. Characterization of an *espI*::Tn mutant

(A) Schematic representation of the position of the transposon insertion in the *espI*::Tn mutant. **(B)** Immunoblots of cell lysates (CL) at 5 μg/well and culture filtrates (CF) at 10 μg/well of wild-type *M. tuberculosis* Erdman (WT) and *espI*::Tn grown in Sauton's medium without Tween-80 for 5 days. Antibodies used are indicated. **(C)** Cytotoxicity assay in THP-1 cells infected with wild-type *M. tuberculosis* Erdman (Erd WT), *espI*::Tn mutant and 5′ Tn::*pe35* at MOI of 5. Data represent the means and standard deviation of at least 4 independent experiments. The y-axis indicates cytotoxicity values relative to the uninfected control. Significance in difference was calculated using Student's *t*-test.

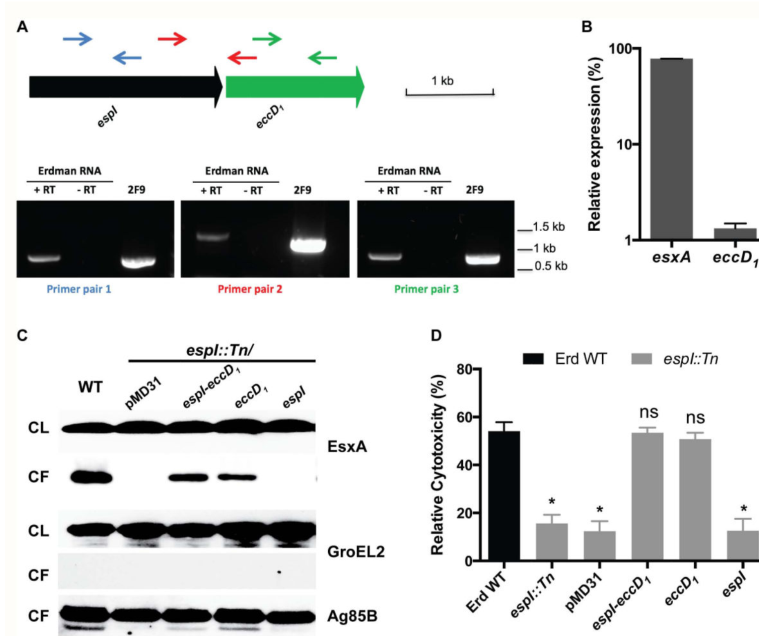

Fig. 2. Complementation of the *espI*::Tn mutant

(A) RT-PCR analysis of *espI* and *eccD₁* cotranscription. Upper panel shows genetic arrangement of *espI* and $eccD_1$, and primer annealing sites. Blue arrows represent the internal primers for *espI*, green arrows the internal primers for $eccD_1$ and red arrows the primers spanning the two genes. Lower panel shows RT-PCR analysis of total RNA from wild-type *M. tuberculosis* Erdman. 2F9 cosmid was used as a positive PCR control. - RT, no reverse transcriptase. **(B)** Quantitative RT-PCR analysis of mRNA levels of *esxA* and $eccD_1$. Relative expression levels were calculated using the Ct method, normalizing transcript levels *to sigA* signals. Data are shown as percentage relative to wild-type *M. tuberculosis.* **(C)** Immunoblots of cell lysates (CL) at 5 μg/well and culture filtrates (CF) at 10 μg/well of wild-type *M. tuberculosis* Erdman (WT) and *espI*::Tn transformed with indicated plasmids grown in Sauton's medium without Tween-80 for 5 days. Antibodies used are indicated. **(D)** Cytotoxicity assay in THP-1 cells infected with wild-type *M. tuberculosis* Erdman (Erd WT) (black bar) and *espI*::Tn (grey bars) transformed with indicated plasmids at MOI of 5. Data represent the means and standard deviation of at least 4 independent experiments. The y-axis indicates cytotoxicity values relative to the uninfected control. Significance in difference compared to wild-type *M. tuberculosis* was calculated using Student's *t*-test. *, $p < 0.0001$; ns, no significant difference.

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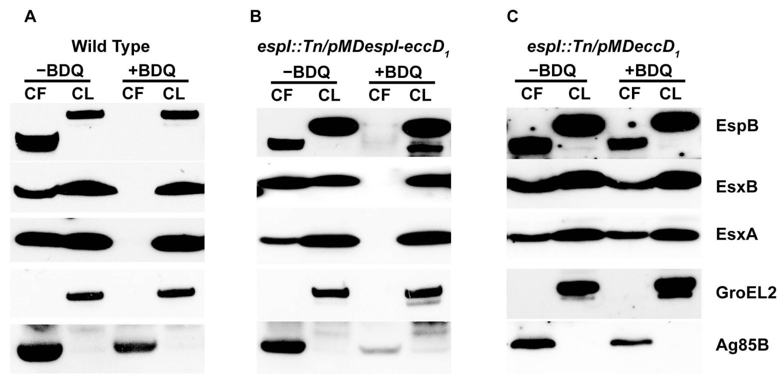

Fig. 3. Effect of BDQ treatment on ESX-1 secretion

Immunoblots of cell lysates (CL) at 5 μg/well and culture filtrates (CF) at 10 μg/well of **(A)** wild-type *M. tuberculosis* Erdman strain, **(B)** *espI::*Tn transformed with pMD*espI-eccD$_1$*, and **(C)** *espI::*Tn transformed with pMD*eccD$_1$* grown in Sauton's medium without Tween-80 and with or without addition of BDQ (0.03 μg/ml) for 5 days. Antibodies used are indicated.

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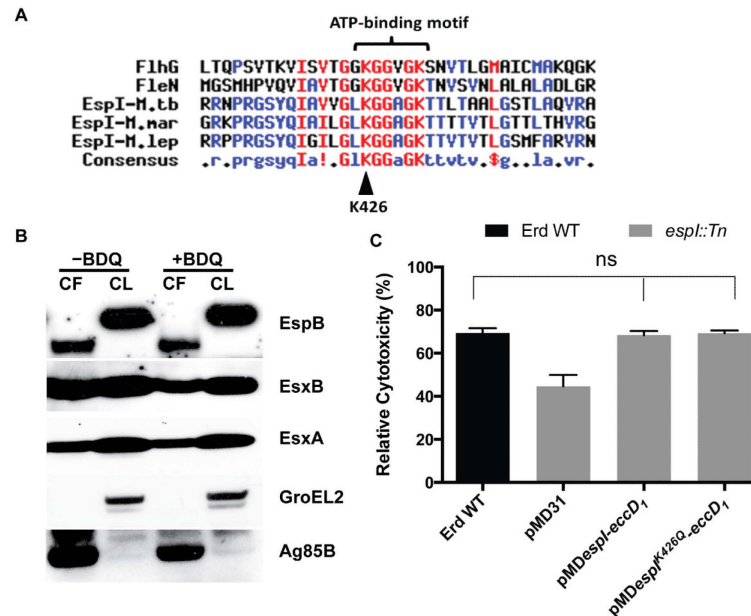

Fig. 4. Impact of BDQ treatment on ESX-1-mediated secretion by the EspI^{K426Q}-expressing strain

(A) Multiple protein sequence alignment of amino acid sequences of *V. cholerae* O1 FlhG (residue 12 to 48), *P. aeruginosa* PAO1 FleN (residue 4 to 40), *M. tuberculosis* EspI (residue 410 to 446), *M. marinum* EspI (residue 623 to 659) and *M. leprae* EspI (residue 330 to 366). The lysine residue, K426, mutagenized and characterized in this study is indicated by a black arrow. **(B)** Immunoblots of cell lysates (CL) at 5 μg/well and culture filtrates (CF) at 10 μg/well of *espI*::Tn transformed with pMD*espI^{K426Q}-eccD$_1$* grown in Sauton's medium without Tween-80 and treated with BDQ (0.03 μg/ml) for 5 days. **(C)** Cytotoxicity assay in THP-1 cells infected with wild-type *M. tuberculosis* Erdman (Erd WT) (black bar), *espI*::Tn (grey bars) transformed with indicated plasmids at MOI of 5. Data represent the means and standard deviation of at least 4 independent experiments. The y-axis indicates cytotoxicity values relative to the uninfected control. Significance in difference was calculated using Student's *t*-test. ns, no significant difference.