A heterodimer of EsxA and EsxB is involved in sporulation and is secreted by a type VII secretion system in *Streptomyces coelicolor*

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An *esx* locus, related to the multiple *esx* loci of *Mycobacterium tuberculosis*, is conserved in all sequenced *Streptomyces* genomes, where it is associated with the developmental regulatory gene *bldB*. Here we demonstrate that the *esxBA* operon, comprising part of the locus, has a novel morphogenetic function in the model species *Streptomyces coelicolor*. This operon encodes two proteins belonging to the WXG-100 superfamily that can form a heterodimer and are secreted in the absence of signal sequences. A mutation in *esxBA* results in a delay in sporulation, with eventual development of aerial hyphae with chains of abnormally sized spore compartments possessing irregular DNA contents. During early sporulation, expression of the operon is elevated in a *bldB* mutant. Other genes in the locus, notably *SCO5734* and *SCO5721*, encode components of a type VII secretion system. Disruption of either of these genes prevents secretion of EsxAB but has no effect on sporulation. To explain the morphogenetic function of EsxAB, we propose that the heterodimer sequesters a regulator of expression of genes involved in nucleoid organization during sporulation.

Received 9 December 2009

Revised 5 March 2010

Accepted 8 March 2010

INTRODUCTION

Gram-positive *Streptomyces* typically inhabit terrestrial soils and marine sediments as free-living saprophytes. In a similar manner to a filamentous fungus, a streptomycete colonizes its particulate environment by growing branching multigenomic hyphae that form a ramifying network, enabling the organism to exploit a localized nutrient source. Being non-motile, they achieve dispersal by means of unigenomic spores borne on specialized non-feeding aerial hyphae. The latter grow out of the semi-aqueous environment inhabited by the feeding vegetative hyphae of terrestrial streptomycetes and ultimately undergo multiple coordinated cell division, generating chains of spores (Flardh & Buttner, 2009). The spores can then be dispersed

by physical agents or the activities of motile animals inhabiting the same niche. The programme of morphological development, initiated by growth of aerial hyphae, is controlled by developmental genes that include the *bld* genes: mutations in these genes can prevent aerial growth. Subsequent maturation of aerial hyphae into mature spore chains, which in the case of the model organism *Streptomyces coelicolor* acquire a typical grey pigmentation, is dependent on developmental *whi* genes. The sporulation process depends on multiple synchronised cell division to subdivide a long syncytial aerial compartment into many spore compartments, into each of which a single copy of the linear chromosome is segregated.

Streptomyces are related to other G + C-rich Actinobacteria, including rod-shaped mycobacteria, of which *Mycobacterium tuberculosis* in particular represents a major global human health problem. A key virulence determinant of this bacterium is the *esx-1* locus. This is a highly conserved locus present in several related copies in the genomes of individual mycobacteria species (Cole *et al.*, 1998; Gey Van Pittius *et al.*, 2001). A deletion within the *esx-1* locus is the primary attenuating mutation of the vaccine strain *Mycobacterium bovis* BCG (Behr *et al.*, 1999; Mahairas *et al.*, 1996), and the locus is also deleted in other attenuated members of the *M. tuberculosis* complex, including the vole bacillus

Abbreviations: AFM, atomic force microscopy; fluo-WGA, fluorescein-conjugated wheatgerm agglutinin; Q RT-PCR, quantitative RT-PCR; RD1, region of difference 1.

Two supplementary figures, showing the results of RT-PCR analysis of the *esxAB* operon and alignments of EsxA and EsxB proteins of *Mycobacterium tuberculosis* and *Streptomyces coelicolor*, and three supplementary tables, showing a comparison of proteins encoded by the *esx* locus of *Streptomyces griseus* and *Streptomyces roseosporus*, predicted proteins encoded by the *S. coelicolor* *esx* cluster aligned with orthologues from other streptomycetes, and oligonucleotides used in the study, are available with the online version of this paper.

Mycobacterium microti (Brodin *et al.*, 2002). The deleted region (known in *M. bovis* BCG as region of difference 1; RD1) includes genes encoding EsxA and EsxB (also known as ESAT-6 and CFP-10, respectively), which are small secreted proteins that lack classical signal sequences and which form a heterodimer (Renshaw *et al.*, 2005). Other genes in the locus encode the ESX-1 membrane-bound secretory apparatus, with protein export driven by ATP hydrolysis. For example, both *rv3870* and *rv3871* encode proteins, EccCa and EccCb, respectively (Bitter *et al.*, 2009), belonging to the FtsK-SpoIIIE ATPase family that are required for secretion of EsxA and EsxB (Simeone *et al.*, 2009). The ESX-1 apparatus is the paradigm for bacterial type VII secretion systems (Abdallah *et al.*, 2007).

Genome analyses suggest that a related secretion system is found in other Actinobacteria and in some Firmicutes (Pallen, 2002). For the latter, it is encoded by a conserved eight gene cluster, three of which share homology with elements of a mycobacterial *esx* locus (Burts *et al.*, 2008). For example, *Staphylococcus aureus* EsxA and EsxB proteins share homology with their mycobacterial counterparts, and their secretion is dependent on EssC, an FtsK-SpoIIIE ATPase (Burts *et al.*, 2005). Mutants defective in production of EsxA and EsxB exhibit decreased virulence, with the suggestion that the secretion system has a role in promoting persistent infections. The function of the secretion system in other Actinobacteria is unknown. Although *Corynebacterium diphtheriae* contains a single orthologous locus (Gey Van Pittius *et al.*, 2001), the gene cluster is not confined to pathogens. For example, the genome of free-living *S. coelicolor* also contains a single complete gene cluster containing orthologues of the typical mycobacterial *esx* locus (Gey Van Pittius *et al.*, 2001). Here we demonstrate that SCO5725 (EsxA) and SCO5724 (EsxB) in *S. coelicolor* are novel morphogenetic proteins whose secretion is dependent on functions encoded by at least two other genes in the locus.

METHODS

Bacterial strains and media. *S. coelicolor* A3(2) and *Escherichia coli* strains are listed and described in Table 1. Cloning procedures were performed in *E. coli* JM109, while *E. coli* ET12567/pUZ8002 was used for intergeneric conjugative transfer of plasmid DNA into *Streptomyces* strains (Kieser *et al.*, 2000). Culturing of *E. coli* strains was as recommended by Sambrook *et al.* (1989). *S. coelicolor* strains were grown on the surface of MS (mannitol soya flour), R2YE or NMMP (no additional carbon source added) agar (Kieser *et al.*, 2000) or in submerged culture with Tryptone Soy Broth (Lab M). *Streptomyces* mutant strains (Table 1) were obtained using Tn5062-mutagenized cosmids (Bishop *et al.*, 2004). In the case of the *bldB* mutant DSCO5723, no insertions in this small gene had been identified in the original insertion library. Consequently, plasmid pSA11 was subjected to *in vitro* transposon mutagenesis as described by Bishop *et al.* (2004). Transposon insertions were mapped by DNA sequencing, and a transposant carrying an insertion in the *bldB*-coding sequence at chromosomal position 6 244 029 (*trpSA11*-F11) was conjugated into *S. coelicolor* M145. Exconjugants carrying a chromosomal copy of the disrupted version of *bldB* were selected. The

identity of all mutants was confirmed by Southern blotting (Sambrook *et al.*, 1989).

DNA manipulation and plasmid construction. Standard DNA procedures were performed as described by Sambrook *et al.* (1989). Plasmid pSA1 was obtained by digesting pBlueScript SK II+ with *NotI* and ligating with a *NotI* fragment from cosmid SC3C3.1.H04 containing the apramycin-resistance gene of Tn5062, *bldB* and *esxB*. Conveniently located *Bam*HI sites within the insert and the plasmid backbone permitted removal of all remaining transposon sequences and part of *bldB*, resulting in pSA2. For complementation analysis, the fragment containing *esxAB* was subsequently subcloned as a *Bam*HI–*Eco*RV fragment into the conjugative integrative plasmid pSH152, yielding pSA3. Construction of a C-terminal His-tagged EsxA required the introduction of a *Xho*I site replacing the *esxA* stop codon in pSA2 by site-directed mutagenesis using oligonucleotides EsatMutEnd1 and -2 (Supplementary Table S3), resulting in plasmid pSAXhoI. This plasmid was digested with *Bam*HI and *Xho*I, and the fragment containing *esxAB* was recovered and combined with a *Xho*I–*Sma*I fragment containing the His-tag-coding sequence released from pET26b. The ligated fragment was cloned into pSH152 previously digested with *Bam*HI and *Eco*RV, yielding pSAHis2. Similarly, an *esxB*_{His6} fusion was constructed by introducing a *Xho*I site replacing the *esxB* stop codon by site-directed mutagenesis (oligonucleotides CFPMutEnd1 and -2) on plasmid pSA2, resulting in pSA2m3. The latter was digested with *Eco*RV/*Xho*I and, together with the *Xho*I–*Sma*I fragment from pET26b mentioned above, ligated to pSH152 digested with *Eco*RV. The resulting integrative plasmid encoding *esxB*_{His6} was called pSAHis4.

For mutagenesis of *bldB*, plasmid pSA1 was digested with *Hind*III and the 2426 bp insert subcloned into pCR2.1-TOPO (Invitrogen) to produce plasmid pSA11. Plasmids for two-hybrid studies were constructed as follows. pSA2 was used as a template in combination with oligonucleotide pairs CFPMUT1 and -2 and ESATMUT1 and -2 (Supplementary Table S3) for consecutive site-directed mutagenesis to introduce *Xma*I and *Bam*HI sites that overlapped the *esxB* and *esxA* start codons, respectively, resulting in plasmid pSA2m2. This plasmid was digested with *Xma*I, *Bam*HI and *Xho*I, and the *Xma*I–*Bam*HI *esxB* and *Bam*HI–*Xho*I *esxA* fragments were recovered and ligated to, respectively, *Xma*I/*Bam*HI-digested pBT and pTRG digested with *Bam*HI and *Xho*I, producing plasmids pBTCFP and pTRGEST. To switch bait and target protein fusions, the *esxA* fragment in pTRGEST was excised with *NotI* and *Xho*I and ligated to pBT digested with the same pair of restriction enzymes to produce pBTTEST. Likewise, the *esxB* fragment in pBTCFP was excised with *Bam*HI and *Xho*I and ligated with pTRG cut with the same enzymes to produce pTRGCFP. Total DNA from *M. tuberculosis* H37Rv was used as template for the PCR amplification of *esxB*_{Mtb} and *esxA*_{Mtb} using the oligonucleotide primer pairs TbCFPF1/TbCFPR1 and TbESATF1/TbESATR1 (Supplementary Table S3). The resulting PCR products were digested with *Eco*RI and *Bam*HI and cloned into pBT and pTRG plasmids digested with the same enzyme combination to produce pBTbesxB and pTTbesxA.

RNA isolation and quantitative RT-PCR (Q RT-PCR). Total RNA isolation and RT-PCR/Q RT-PCR procedures were performed as previously described (Del Sol *et al.*, 2006; Fernandez-Martinez *et al.*, 2009). Briefly, sterile cellophane disks placed on top of MS plates were inoculated with *Streptomyces* spores. Disks remained in place for a specified time, after which mycelia were collected and total RNA was isolated with a Qiagen RNeasy Mini kit. cDNAs were obtained using a RETROscript reverse transcription kit (Ambion) with random decamers. Q RT-PCR was carried out on an iCycler iQ real-time PCR detection system (Bio-Rad) using SYBR Green Supermix 2× containing Thermo-Start DNA polymerase (Abgene). Gene-specific primers (Supplementary Table S3) were designed using Beacon

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description or transposon insertion (genome position)	Source or reference
Strains		
<i>S. coelicolor</i> M145	SCP1 [−] , SCP2 [−]	Kieser <i>et al.</i> (2000)
<i>E. coli</i> JM109	F' <i>traD36 proA</i> + B+ <i>lacIq</i> Δ(<i>lacZ</i>)M15/Δ(<i>lac-proAB</i>) <i>glnV44 e14[−] gyrA96 recA1 relA1 endA1 thi hsdR17</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>E. coli</i> ET12567 (pUZ8002)	<i>dam13::Tn9 dcm6 hsdM hsdR recF143 zjj201::Tn10 galK2 galT22 ara14 lacY1 xyl5 leuB6 thi1 tonA31 rpsL136 hisG4 tsx78 mtli glnV44</i> , containing the non-transmissible <i>oriT</i> mobilizing plasmid pUZ8002	Flett <i>et al.</i> (1997)
<i>E. coli</i> BacterioMatch Reporter Strain	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [F' <i>laqIq bla lacZ Kan</i>]	Stratagene
DSCO5721	M145 <i>SCO5721::Tn5062</i> , Tn insertion SC3C3.2.B06 (6 241 534)	This study
DSCO5722	M145 <i>SCO5722::Tn5062</i> , Tn insertion SC3C3.1.G11 (6 242 898)	This study
DSCO5723	M145 <i>SCO5723::Tn5062</i> , Tn insertion <i>tnpSA11-F11</i> (6 244 029)	This study
DSCO5724	M145 <i>SCO5724::Tn5062</i> , Tn insertion SC3C3.1.H11 (6 244 769)	This study
DSCO5734	M145 <i>SCO5734::Tn5062</i> , Tn insertion SC3C3.2.B11 (6 256 849)	This study
DSCO5735	M145 <i>SCO5735::Tn5062</i> , Tn insertion SC3C3.2.F08 (6 257 786)	This study
Plasmids		
pBlueScript SK II +	Cloning vector	Stratagene
pSA1	pBlueScript SK II + with <i>bldB esxB</i> A	This study
pSA2	pBlueScript SK II + with <i>esxB</i> A	This study
pSH152	<i>hyg</i> derivative of pSET152	Mistry <i>et al.</i> (2008)
pSA3	pSH152 with <i>esxB</i> A	This study
pSAXhoI	pSA2-based, <i>XhoI</i> site replacing <i>esxA</i> stop codon	This study
pET26b	Cloning vector	Novagen
pSAHis2	pSH152 derivative, <i>esxB</i> _{[His]6}	This study
pCR2.1-TOPO	Cloning vector	Invitrogen
pSA11	pCR2.1-TOPO with <i>bldB esxB</i> A	This study
pSA2m1	pSA2-based, <i>XmaI</i> site overlapping <i>esxB</i> start codon	This study
pSA2m2	pSA2m1-based, <i>BamHI</i> site overlapping <i>esxA</i> start codon	This study
pSA2m3	pSA2-based, <i>XhoI</i> site replacing <i>esxB</i> stop codon	This study
pSAHis4	pSH152 derivative, <i>esxB</i> _{[His]6}	This study
pBT	Bacteriomatch two-hybrid vector	Stratagene
pTRG	Bacteriomatch two-hybrid vector	Stratagene
pBTCFP	pBT containing <i>S. coelicolor esxB</i>	This study
pTRGEST	pTRG containing <i>S. coelicolor esxA</i>	This study
pBTTEST	pBT containing <i>S. coelicolor esxA</i>	This study
pTRGCFP	pTRG containing <i>S. coelicolor esxB</i>	This study
pBTbesxB	pBT vector containing <i>M. tuberculosis esxB</i> (Rv3874)	This study
pTTbesxA	pTRG vector containing <i>M. tuberculosis esxA</i> (Rv3875)	This study

Design 2.0 (Premier Biosoft). Dilutions of *S. coelicolor* genomic DNA of known concentration were used as standards and total RNA samples were included as negative controls. The *S. coelicolor hrdB* gene, encoding the principal sigma factor, was used as an internal control.

Protein methods. Strains carrying pSAHis2 were grown in Tryptone Soy Broth until mid-exponential phase (around 24 h at 30 °C, 250 r.p.m.). Complete Protease Inhibitor (Roche) was added to the culture and cells were collected by centrifugation. The supernatant was treated with 2 % TCA at 4 °C for 1 h, followed by centrifugation at 40 000 g for 20 min. The resulting pellet was washed twice with ice-cold acetone and left to air dry. Sonication buffer (50 mM Tris/HCl, pH 8.0, 200 mM NaCl, 15 mM EDTA) was added to resuspend precipitated proteins and was subsequently mixed with an equal volume of 2× Laemmli buffer. The protein concentration of the resuspended sample was determined using a 2-D Quant kit (GE Healthcare). Intracellular proteins were released by sonication (20 s

burst on ice) of the cell pellet resuspended in ice-cold sonication buffer. Unbroken cells and insoluble components were removed by centrifugation at 40 000 g, and the resulting supernatant was used to assess soluble intracellular proteins. The concentrations of soluble protein fractions were determined using Bradford reagent (Bio-Rad). Protein samples (5–10 µg total protein) were separated on a 12% SDS-PAGE gel and transferred to Hybond-P (GE Healthcare). His-tagged EsxA was detected using a penta-His-peroxidase conjugate antibody (Qiagen) and an ECL Advance Western blotting detection kit (GE Healthcare) following the manufacturer's recommendations.

Bacterial two-hybrid assays. The BacterioMatch II two-hybrid system (Stratagene) was used to explore protein interactions. Translational fusions were constructed and introduced into the supplied *E. coli* BacterioMatch II two-hybrid system reporter strain. β-Galactosidase assays were performed as recommended by the manufacturer, and for each interaction tested at least four independent clones were analysed and the combined data then averaged.

Assessment of mutant phenotypes. To assess colony morphology, strains were plated on MS agar (Kieser *et al.* 2000). Strains used in fluorescent microscopy were taken as coverslip impressions on the surface of MS growing colonies after 3 days or grown in the acute angle of a coverslip inserted in the agar medium. Cell walls and DNA were stained with fluorescein-conjugated wheatgerm agglutinin (fluo-WGA) and propidium iodide (both from Molecular Probes), respectively, as described by Schwedock *et al.* (1997). Live-cell nucleoids were stained with SYTO9 (Invitrogen) (5 μ M diluted in 20 % v/v, glycerol). Images were obtained using a Nikon Eclipse E600 epifluorescence microscope fitted with a Coolsnap microscope camera (RS Photometrics). Fluorescence intensity profiles along spore chains stained with SYTO9 were created in ImageJ (Abramoff *et al.*, 2004). Briefly, freehand lines, approximately 12 μ m in length, were drawn along the spore chains, passing through the centre of adjacent nucleoids. Fluorescent profiles were created using the 'plot profile' function. Measurements of spore compartment size were performed using Scion Image software (Scion Corp.); at least 500 measurements per strain were performed. Atomic force microscopy (AFM) samples were prepared and imaged as described previously (Del Sol *et al.*, 2007).

RESULTS

Architecture of the conserved *esx* locus in *Streptomyces*

Analysis of sequenced streptomycete genomes for the presence of the minimal *esx* cluster originally identified in *S. coelicolor* (Gey Van Pittius *et al.*, 2001) revealed a single

conserved cluster in five other species (Fig. 1). Certain aspects of the overall architecture of the streptomycete *esx* locus are conserved. In each case the cluster is delimited on one side by two genes that are likely to be co-transcribed and which encode (i) an N-terminal transmembrane protein with an ATP/GTP-binding site, and (ii) a protein related to the mycosin, subtilisin-like cell wall-associated serine protease family. At the other end of the cluster are two divergently transcribed genes that encode (i) an integral membrane protein and (ii) an FtsK-SpoIIIE ATPase. Contained within each cluster is an orthologue of the *esxBA* operon, each gene encoding a protein that belongs to the WXG-100 protein family, members of which are characterized by a central amino acid motif Trp-X-Gly and a size of approximately 100 amino acids (Pallen, 2002). For each species the cluster is linked with the *bldB* gene, which encodes a transcriptional regulator required for aerial development in *S. coelicolor* (Mishig-Ochiriin *et al.*, 2003; Pope *et al.*, 1998). Whereas the *Streptomyces pristinaespiralis* genome contains a minimal uninterrupted locus, for the other species additional ORFs separate different elements of the locus. The overall architecture of the *Streptomyces roseosporus* *esx* locus resembles that of *Streptomyces griseus* and the relatedness of these two species is underlined by the very high degree of identity of the proteins encoded by both clusters (Supplementary Table S1). Alignments of the proteins encoded by the *S. coelicolor* cluster with those of the other species revealed in each case

Fig. 1. Architecture of the conserved minimal *esx* locus in *Streptomyces*. The genes comprising the *esx* cluster of *S. coelicolor* (Gey Van Pittius *et al.*, 2001) were compared with their orthologues in five other sequenced streptomycetes. The locus of *S. roseosporus* (not shown) resembles that of *S. griseus*. The colours of genes indicate the corresponding predicted proteins: N-terminal transmembrane protein with ATP/GTP-binding site, orange; subtilisin-like cell wall-associated serine protease, red; BldB regulatory protein, black; EsxB, light blue; EsxA, blue; FtsK-SpoIIIE ATPase, yellow; integral membrane protein, green.

that the FtsK-SpoIIIE ATPase is the best-conserved component, whereas the two WXG-100 proteins are generally the least well conserved (Supplementary Table S2). As noted elsewhere (Gey Van Pittius *et al.*, 2001), the locus may extend to include SCO5717 (and the equivalent orthologues in the other five streptomycete genomes), an orthologue of *espI* (*rv3876*) that is unique to the *esx-1* locus of *M. tuberculosis*.

Disruption of *esxBA* delays sporulation and affects spore DNA content and morphology

The *esxB* and *esxA* genes are separated by a short 37 bp intergenic region, suggesting their coexpression as an operon. This was confirmed by RT-PCR using as forward primer a sequence from the 5' region of *esxB* (oligonucleotide All3S1, Supplementary Table S3) and as reverse primer a sequence complementary to the 3' sequence of *esxA* (All2SA1, Supplementary Table S3). The expected-size 715 bp amplicon was only obtained when reverse transcriptase was included in the reaction (Supplementary Fig. S1a). *esxA* is separated from the next ORF (SCO5726) by a 120 bp intergenic region. No amplicon was obtained after RT-PCR using the same forward primer and a reverse primer complementary to the 3' sequence of SCO5726 (All3SA1, Supplementary Table S3), although the expected 1.3 kb amplicon was obtained using chromosomal DNA as template (Supplementary Fig S1b). Hence we conclude that SCO5726 is transcribed independently from the *esxBA* operon.

To investigate the function of the *S. coelicolor* EsxA and EsxB proteins, a polar mutant, DSCO5724, with a Tn5062 insertion in *esxB* was constructed. The polarity was confirmed by RT-PCR: using the 5' *esxB* forward primer and 3' *esxA* reverse primer, an expected 4.2 kb amplicon was obtained with mutant genomic DNA as template, but not with mutant RNA (results not shown). To address the question of whether *esxA* is transcribed independently from a second promoter within the operon, another RT-PCR was performed with a forward primer complementary to the 5' region of *esxA* (EsxAFOR, Supplementary Table S3) and the 3' *esxA* reverse primer. With RNA from M145, an expected 194 bp amplicon was obtained, but no amplicon was detected with template RNA from the mutant DSCO5724 (results not shown), suggesting that, with the growth conditions employed, there was no evidence for an *esxA*-specific promoter. Compared with the wild-type M145 strain, the mutant exhibited delayed sporulation on MS medium (Fig. 2a). Whereas M145 sporulated after 36 h incubation, the mutant required an extra 2–3 days' incubation to reach an equivalent developmental maturity. A delay in sporulation was also evident on several other media tested, including R2YE and NMMP agar (results not shown). To link this developmental phenotype with the *esxBA* operon, a mutant with an insertion in the immediate downstream gene, SCO5726, was analysed. This mutant underwent sporulation within

the same incubation period as the wild-type (data not shown). Introduction of either of the complementing plasmids pSA3 or pSAHis2, each with an intact copy of the *esxBA* operon, restored the ability of the *esxBA* mutant to develop normally (Fig. 2a).

The developmental phenotype exhibited by the *esxBA* mutant prompted microscopic analysis of its spore chains. The aerial hyphae were examined after fluo-WGA staining to reveal cell walls defining spore compartments. Compared with the regularity of spore compartments (mean length 1.3 μ m) in the aerial hyphae of M145, the mutant possessed a significantly higher proportion of large compartments of length >1.9 μ m (Fig. 2b, Table 2). Live DNA staining of septated aerial hyphae indicated that the aberrant-sized compartments of the mutants correlated with irregular DNA content (Fig. 3). The large compartments of the *esxBA* mutant appeared to contain multiple nucleoids. While these aberrant compartment sizes with irregular DNA contents are indicative of a role of the *esxBA* operon in correct septation, additionally we examined the topography of the surface of the spore chains using AFM. The topographic height images and, in particular, the derived corresponding 3D representations revealed a characteristic abnormal 'wrinkled' morphology of the spore compartments of the *esxBA* mutant, in contrast to the relatively smooth surface topography of wild-type spore compartments (Fig. 2c). AFM at higher-level magnification revealed a normal rodlet layer on the surface of the mutant (data not shown). Spore chains of mutants with either pSA3 or pSAHis2 complementing plasmids resembled the wild-type M145 strain when examined by either epifluorescence microscopy or AFM (data not shown).

EsxB and EsxA of *S. coelicolor* form a complex

An alignment of *S. coelicolor* EsxA with EsxA of *M. tuberculosis* revealed 13 % identity and 29 % similarity, and alignment of the two EsxB proteins showed 12 % identity and 34 % similarity (Supplementary Fig. S2). In addition to the conserved WXG motifs, amino acid residues believed to stabilize a mini-hydrophobic core within *M. tuberculosis* EsxA are well conserved (Lightbody *et al.*, 2008). In addition, there is good conservation of critical hydrophobic residues within the α -helices that are implicated as lying at intra- and intermolecular interfaces of the four-helix bundle structure formed by the complex of *M. tuberculosis* EsxA and EsxB (Lightbody *et al.*, 2008).

Based on these alignments it could be expected that, similar to *M. tuberculosis* EsxA and EsxB, the equivalent *S. coelicolor* proteins would form a 1:1 heterodimeric complex. To test this we employed a bacterial two-hybrid system (Karimova *et al.*, 1998), creating translational fusions between the bait protein and λ repressor, and the target protein and the N-terminal domain of RNA polymerase. With this system a strong interaction between *M. tuberculosis* EsxA and EsxB was confirmed, and an

Fig. 2. Delayed sporulation and aberrant spore compartments due to disruption of *esxB*. (a) A polar *esxBA* insertion mutant exhibits delayed sporulation compared with the parental M145 strain grown on MS medium for 36 h. (b) Aerial hyphae from 72 h cultures were stained with Fluo-WGA to reveal sporulation septa; the arrow points to an unusually long spore compartment in the *esxBA* mutant (bar, 5 μ m). (c) AFM topographic height images (H) and corresponding 3D representations (3D) of spore chains of the parental strain and mutant revealing a 'wrinkled' morphology of the latter.

equivalent binding between *S. coelicolor* EsxA and EsxB was measured (Table 3). Similar results were obtained after switching bait and target protein fusions. We also assessed the specificity of complex formation by combining translational fusions of EsxA and EsxB from the two different Actinobacteria: for these combinations we could not detect any interactions. Moreover, as for *M. tuberculosis* EsxA and EsxB (Lightbody *et al.*, 2004), we could not detect homodimer formation for *S. coelicolor* EsxA and EsxB.

The *S. coelicolor* *esx* locus encodes a type VII secretion system

The ESX-1 system of *M. tuberculosis* responsible for secretion of EsxA and EsxB, which lack classical signal sequences, is termed a type VII secretion system (Abdallah *et al.*, 2007). To investigate secretion of *S. coelicolor* EsxA, an additional copy of the operon under the control of the native promoter but expressing a His₆-tagged version of the protein was expressed in M145. In a time-course

experiment, EsxA[His]₆ was detected in both culture supernatants and cytoplasmic fractions after 16 h incubation, reaching maximum abundance after 24 h but then declining, so that very little could be detected after 40 h incubation (Fig. 4a). A similar expression profile was observed when a tagged version of *esxB* controlled by the native promoter was introduced into M145: EsxB[His]₆ was detected in the supernatant of 24 h cultures, but little or no protein was detected at 40 h (Fig. 4a). Based on this result, we then examined localization of EsxA[His]₆ by expressing an additional copy of the operon in different genetic backgrounds after 24 h incubation. EsxA[His]₆ was detected in the culture supernatants of three mutants with the integrated tagged operon: DSCO5724 with pSAHis2, the complemented *esxBA* mutant, DSCO5722 with pSAHis2, carrying an insertion in a predicted serine protease gene *SCO5722* (Fig. 4b), and DSCO5735 with pSAHis2, disrupted in the integral membrane protein gene *SCO5735* (Fig. 4c). However, EsxA[His]₆ was not detected in the culture supernatants of either DSCO5734 or DSCO5721 containing pSAHis2, mutants with insertions

Table 2. Distribution of pre-spore compartment sizes

Strain	Percentage of spores of size:		
	<0.8 μm	0.8–1.9 μm	>1.9 μm
M145	0.97	95.87	3.15
DSCO5724 (<i>esxB::Tn5062</i>)	0.4	86.16	13.43

in genes encoding, respectively, an FtsK/SpoIIIE-type protein and an N-terminal transmembrane protein with a likely ATP/GTP-binding domain. EsxA[His]₆ was an easily detectable cytoplasmic protein in both these mutants (Fig. 4b). These results indicate that secretion of EsxA is dependent on the activity of two genes in the *esx* locus that are homologous to genes encoding components of the ESX type VII secretion system of *M. tuberculosis*. Interestingly, apart from the *esxBA* mutant, none of the other mutants of the *S. coelicolor* ESX-1 locus exhibited delayed sporulation (results not shown).

Expression of the *esxBA* operon is regulated by BldB

The operon is divergently transcribed from *bldB*, a gene known to control aerial development. BldB represses its own expression (Pope *et al.*, 1998) and can bind to a 76 bp region from the 304 bp intergenic region that separates *bldB* and *esxB* (Mishig-Ochiriin *et al.*, 2003). The presence of *bldB* within the *esx* locus prompted investigation of whether BldB also regulates expression of the *esxBA* operon. Q RT-PCR of RNA purified at different time points revealed a fourfold increase in expression of *esxBA* at 36 h in the *bldB* mutant strain DSCO5723 compared with M145 (Fig. 5).

To check whether the developmental phenotype of the *esxBA* mutant results from an effect of the *esxAB* mutation on *bldB* expression, we performed a time-course analysis of *bldB* mRNA abundance, comparing M145 and the *esxBA* mutant. We found no significant differences between the strains, with increasing abundance observed from 36 h onwards, in agreement with the expression profile for *bldB* reported by Pope *et al.* (1998), indicating that the defect in sporulation and, in particular, nucleoid segregation is indeed due to loss of function of *esxBA*.

DISCUSSION

We have provided experimental evidence for a type VII secretion system in the model streptomycete *S. coelicolor*. The ESX-1 pathway of *M. tuberculosis* is the paradigm for these secretion systems in bacteria as, due to its critical role in virulence, it has been the subject of intense analysis. The ESX-1 system has been implicated in innate immune modulation (MacGurn & Cox, 2007; Stanley *et al.*, 2003, 2007; Volkman *et al.*, 2004), especially early after infection in macrophages, although the precise role of the major secreted substrates and key effector molecules EsxA (ESAT-6) and EsxB (CFP-10) is still debated. At least five genes encoded within the approximately 15-gene *esx-1* cluster, *Rv3868* to *Rv3871* and *Rv3877*, are required for the secretion of these substrates (Brodin *et al.*, 2006; Guinn *et al.*, 2004; Hsu *et al.*, 2003; Stanley *et al.*, 2003). *Rv3877* encodes an integral membrane protein, EccD, that may form all or part of a channel. *Rv3869* also encodes a membrane-associated protein. Secretion is believed to be driven by ATP hydrolysis: *Rv3870* and *Rv3871* encode ATPases, EccCa and EccCb, respectively, belonging to the FtsK-SpoIIIE family, and *Rv3868* encodes another ATPase, EccA.

Fig. 3. Fluorescence profiles of SYTO9-stained nucleoids. Images used to determine the profiles are shown next to the relative fluorescence plotted along a 10–13 μm line bisecting the stained nucleoids. Bars, 4 μm .

Table 3. Interactions between EsxA and EsxB detected using a bacterial two-hybrid system

Figures in parentheses are β -galactosidase units per milligram bacterial dry weight. –, No interaction; ++, strong interaction.

Target	Bait			
	EsxA _{Mt}	EsxA _{Sc}	EsxB _{Mt}	EsxB _{Sc}
EsxA _{Mt}	– (125)	– (131)	++ (6585)	– (140)
EsxA _{Sc}	– (136)	– (113)	– (108)	++ (7241)
EsxB _{Mt}	++ (5684)	– (134)	– (145)	– (123)
EsxB _{Sc}	– (151)	++ (7013)	– (134)	– (121)

The *esx* locus in *S. coelicolor* was originally predicted by comparative genome analysis with the *esx* clusters of *M. tuberculosis* (Gey Van Pittius *et al.*, 2001). Indeed, it most closely resembles the *esx-4* locus that phylogenetic analysis indicates to be the ancestral mycobacterial locus. The *esx-4* locus also encodes the least number of proteins, with for example a single FtsK-SpoIIIE orthologue, EccC, rather than the function provided by the two smaller 'split' orthologues, EccCa and EccCb, encoded by the *esx-1* locus, although no functional analysis of the *esx-4* genes has been reported. In *S. coelicolor*, secretion of both EsxA and EsxB

substrates is dependent on the function of the predicted FtsK-SpoIIIE ATPase encoded by *SCO5374* and the membrane-associated protein encoded by *SCO5721* (an orthologue of *Rv3869*), but is independent of the function of either the serine protease encoded by *SCO5722* or the integral membrane protein *SCO5735* (an orthologue of *Rv3877*). Although we cannot exclude that other genes neighbouring or unlinked to the cluster may provide additional functions for the *Streptomyces* type VII secretion system, our initial analysis indicates that in terms of its complexity the machinery has fewer components compared with the mycobacterial ESX-1 system. In this respect it may be related to the simpler type VII secretion system of *Staph. aureus*, which requires the function of the FtsK-SpoIIIE protein EssC and two smaller predicted membrane proteins, EssA and EssB, for secretion of the substrates EsxA and EsxB (Burts *et al.*, 2005). Other genes in the *S. aureus* *ess* cluster, for example *esaA*, which encodes an integral membrane protein, are dispensable for secretion of the substrates.

In *M. tuberculosis*, an interaction between a specific C-terminal seven amino acid signal of EsxB and *Rv3871* is necessary for secretion of the former (Champion *et al.*, 2006). As secretion of EsxA is dependent on functional EsxB, a model for secretion of these substrates posits that

Fig. 4. Secretion of EsxA_{[His]6} and EsxB_{[His]6}. (a) EsxA_{[His]6} (left-hand panel) or EsxB_{[His]6} (right-hand panel) were detected by immunoblotting in the supernatants of cultures of M145 containing pSAHis2 or pSAHis4 grown for the indicated time period (10 µg total protein per lane). (b, c) To investigate the dependence of EsxA_{[His]6} secretion on other functions encoded by the *esx* locus, strains containing pSAHis2 [except lane 2 in (b), with the empty vector pSH152] were grown for 24 h and the presence of EsxA_{[His]6} was analysed in both extracellular and intracellular fractions: (b) lane 1, M145, extracellular fraction; (b) lane 2, M145, extracellular fraction; (b) lane 3/ (c) lane 1, DSCO5724 (*esxB*::Tn5062), extracellular fraction; (b) lane 4, DSCO5734 (*SCO5734*::Tn5062), extracellular fraction; (b) lane 5, DSCO5722 (*SCO5722*::Tn5062), extracellular fraction; (b) lane 6, DSCO5721 (*SCO5721*::Tn5062), extracellular fraction; (b) lane 7, DSCO5734 (*SCO5734*::Tn5062), intracellular fraction; (b) lane 8, DSCO5722 (*SCO5722*::Tn5062), intracellular fraction; (b) lane 9, DSCO5721 (*SCO5721*::Tn5062), intracellular fraction; (c) lane 2, DSCO5724 (*esxB*::Tn5062), intracellular fraction; (c) lane 3, DSCO5735 (*SCO5735*::Tn5062), extracellular fraction; (c) lane 4, DSCO5735 (*SCO5735*::Tn5062), intracellular fraction (5 µg total protein per lane).

Fig. 5. Regulation of *esxBA* by BldB. Q RT-PCR analysis of the abundance of *esxB* (SCO5724) mRNA in the parental strain M145 and a *bldB* mutant, DSCO5723. The strains were grown on MS medium and RNA was purified at the times indicated. The data were normalized using *hrdB* mRNA as a control. The data were obtained from two biological replicates with three technical replicates per biological replicate for each time point.

the cytoplasmic proteins form a stable heterodimer and that the heterodimer is targeted for secretion by interaction between the signal in the unstructured C-terminal domain of EsxB and the FtsK-SpoIIIE protein. Although our data indicate that *S. coelicolor* EsxA and EsxB likewise form a heterodimer, there is no obvious amino acid sequence homology at the C terminus of the EsxB protein with the signal sequence of the *M. tuberculosis* protein (Supplementary Fig. S2). Although this signal is conserved in other EsxB orthologues (e.g. *Staph. aureus* EsxB), it is also absent in mycobacterial paralogues such as EsxU encoded by the *esx-4* locus. It remains to be determined how the *S. coelicolor* heterodimer is targeted for secretion.

Loss of function of the EsxAB heterodimer impacts on sporulation. The *esxBA* mutant has irregular-sized pre-spore compartments with corresponding aberrant DNA contents; there is an increased abundance of large compartments that apparently contain multiple nucleoids. This implies a loss of coordination of cell division with segregation of nucleoids. Orchestration of multiple synchronous cell division with compaction and segregation of individual nucleoids in aerial hyphal filaments is a complex albeit poorly understood process. Several functions have been identified that influence the outcome. The *S. coelicolor* SMC protein is associated with the nucleoid at specific foci during early stages of sporulation septation, and disruption of *smc* results in less-condensed nucleoids and a higher frequency of anucleate spore compartments (Kois *et al.*, 2009). The defects in segregation are enhanced by combining *smc*, *ftsK* and *parB* mutations (Dedrick *et al.*, 2009), confirming the functional interplay between nucleoid-condensing (SMC) and segregosome elements (FtsK, ParB). But even in a triple *ftsK smc parB* mutant, the majority of spores contain complete nucleoids, implying that coordination of nucleoid condensation and segregation with synchronous multiple septation in an aerial filament is dependent on several overlapping and partially redundant functions. Apparently the EsxAB heterodimer also has a fundamental role in the process, although the frequency of anucleate compartments produced by the

mutants does not differ from the wild-type frequency (results not shown). The irregular-sized pre-spore compartments with corresponding aberrant DNA contents of the *esxBA* mutant resemble the phenotype of an *S. coelicolor* *dpsA* mutant (Facey *et al.*, 2009). The Dps (DNA protection in starved cells) proteins in *Streptomyces* play an important role in the massive topological restructuring of nucleoids during sporulation, when the diffuse nucleoids of vegetative filaments become highly condensed to permit the subsequent segregation of one nucleoid per pre-spore compartment. We believe that the EsxAB heterodimer could regulate this process and propose a model whereby the heterodimer functions to sequester one or more factors that function to regulate correct nucleoid condensation. Due to the relatively low abundance of EsxAB (as estimated from Western blots), this factor is less likely to be an abundant structural protein associated with the nucleoid, and more likely to be a key regulator of expression of nucleoid-associated proteins. A precedent for an interaction between EsxAB and the regulatory protein EspR is provided in *M. tuberculosis* (Raghavan *et al.*, 2008). EspR is a transcription factor that regulates expression of ESX-1 and is secreted by this system in an EsxAB-dependent manner. Sequestration of a regulatory protein that acts during vegetative growth as a brake to prevent nucleoid restructuring would represent a commitment to sporulation, and this would be reinforced by its subsequent secretion via the ESX system. SCO6675, an EspR orthologue in *S. coelicolor*, is a candidate regulator whose function we are currently investigating. Alternatively, BldB could be sequestered. This is a critical pleiotropic morphogenetic regulator linked to the ESX-1 locus in many sequenced streptomycetes; we have shown that the *esxBA* operon is either directly or indirectly repressed by BldB during early sporulation. Interestingly, overexpression of BldB, which could have an impact on *esxBA* expression, has been shown to block sporulation after development of aerial hyphae, indicating that levels of this morphogen are critical for correct progression of the developmental programme (Pope *et al.*, 1998).

While the ESX-1 locus is the major virulence determinant of *M. tuberculosis* it also influences bacterial colony morphology. A change in colony morphology was observed in the initial passaging of *M. bovis* to obtain the BCG vaccine strain (Calmette, 1927). Complementation of BCG with RD1 DNA reverses this change to large extent and, similarly, addition of this region to an *M. microti* vaccine strain alters its colony morphology (Pym *et al.*, 2002). For both these mycobacterial vaccine strains, the change in colony morphology has also been reported when only a subfragment of RD1 is introduced that is insufficient to restore all necessary functions of the type VII secretion system. The subfragment includes the *esxAB* operon and two immediate upstream genes not considered critical components of the secretion system. This suggests that the intracellular EsxAB heterodimer influences mycobacterial cell structure, in addition to the virulence function of the secreted complex. This parallels our observations in *S. coelicolor*. An important intracellular morphogenetic function of the heterodimer in *S. coelicolor* is indicated by the difference between the delayed sporulation phenotype of *esxBA* mutants and the normal sporulation exhibited by the secretion mutants. Clearly, this intracellular role in actinomycetes, possibly involving sequestration of one or more morphogenetic regulators as discussed, warrants further investigation.

ACKNOWLEDGEMENTS

This research has been supported by grants from the UK Biotechnology and Biological Sciences Research Council (BBSRC) (BB/E019242/1) and the European Commission (LSH-IP 005224 and ALFA II-01313-FA-FCO).

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Edited by: M. S. Paget