The ESX/type VII secretion system modulates development, but not virulence, of the plant pathogen *Streptomyces scabies*

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SUMMARY

Streptomyces scabies is a model organism for the investigation of plant–microbe interactions in Gram-positive bacteria. Here, we investigate the type VII protein secretion system (T7SS) in *S. scabies*; the T7SS is required for the virulence of other Gram-positive bacteria, including *Mycobacterium tuberculosis* and *Staphylococcus aureus*. The hallmarks of a functional T7SS are an EccC protein that forms an essential component of the secretion apparatus and two small, sequence-related substrate proteins, EsxA and EsxB. A putative transmembrane protein, EccD, may also be associated with T7S in Actinobacteria. In this study, we constructed strains of the plant pathogen *S. scabies* carrying marked mutations in genes coding for EccC, EccD, EsxA and EsxB. Unexpectedly, we showed that all four mutant strains retain full virulence towards several plant hosts. However, disruption of the *esxA* or *esxB*, but not *eccC* or *eccD*, genes affects *S. scabies* development, including a delay in sporulation, abnormal spore chains and resistance to lysis by the *Streptomyces*-specific phage ϕ C31. We further showed that these phenotypes are specific to the loss of the T7SS substrate proteins EsxA and EsxB, and are not observed when components of the T7SS secretion machinery are lacking. Taken together, these results imply an unexpected intracellular role for EsxA and EsxB.

INTRODUCTION

Secreted proteins play a number of essential roles in bacteria, including the colonization of niches and host–pathogen interactions. Gram-negative bacteria, which have double-membrane cell envelopes, exhibit six major protein secretion pathways (types I–VI) that move proteins from the cytoplasm to the cell exterior by a one- or two-step mechanism (reviewed in Desvaux *et al.*, 2009). These secretion systems are absent from Gram-positive bacteria, which generally (with the exception of mycobacteria and closely related genera) have a simpler cell envelope organization comprising a single membrane. The Sec and Tat pathways, which are located in the cytoplasmic membrane of Gram-negative and

Gram-positive bacteria, directly secrete extracellular proteins in Gram-positive bacteria (e.g. Sibbald *et al.*, 2006; Widdick *et al.*, 2006). In addition to these general export pathways, Gram-positive bacteria possess a specialized protein export system, termed the type VII or ESX secretion system (Burts *et al.*, 2005; Hsu *et al.*, 2003; Pym *et al.*, 2003; Stanley *et al.*, 2003).

The type VII secretion system (T7SS) was initially described in mycobacteria, where it was demonstrated that a secretion system was encoded by the region of difference 1 (RD1) of *Mycobacterium bovis* that was required for the secretion of the small proteins ESAT-6 and CFP-10 (now renamed EsxA and EsxB, respectively; Hsu *et al.*, 2003; Pym *et al.*, 2003). These small proteins lack N-terminal signal sequences and are structurally closely related, comprising a helical hairpin with a conserved W–x–G motif at the hairpin bend (Renshaw *et al.*, 2005; Sundaramoorthy *et al.*, 2008). The presence of one or more genes encoding an EsxA family protein is one of the genetic hallmarks for a T7SS, together with a gene coding for an ATPase of the FtsK-SpoIIIE family, which is required for EsxA secretion (Brodin *et al.*, 2006; Burts *et al.*, 2005; Stanley *et al.*, 2003). The T7SS is found throughout the Actinobacteria and also in the Firmicutes. However, the similarity between the secretion systems of the Firmicutes and Actinobacteria is very low, with the EsxA/B proteins and the FtsK-SpoIIIE domain protein being the only conserved elements. In addition, there are a number of system-specific components that are essential for T7S in the different phyla. The T7SS is required for full virulence of a number of Gram-positive pathogens, including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Staphylococcus aureus* and *Bacillus anthracis* (Burts *et al.*, 2005; Garufi *et al.*, 2008; Hsu *et al.*, 2003; Pym *et al.*, 2003; Stanley *et al.*, 2003).

Streptomyces forms the largest genus of Actinobacteria and contains mycelial organisms that undergo complex morphological differentiation involving the formation of aerial hyphae and spores (Flardh and Buttner, 2009). *Streptomyces* spp. are frequently soil dwelling, and are prolific protein secretors, particularly during their vegetative growth stage (Chater *et al.*, 2010). At least one copy of a T7SS is encoded in the genomes of all streptomycetes sequenced to date. *Streptomyces* spp. are predominantly saprophytic, although a few members of the genus are able to cause disease. The best studied of the disease-causing streptomycetes is

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Streptomyces scabies, which is the causal agent of common scab of potato. Thaxtomin, a nitrated dipeptide toxin, is one of the major pathogenicity factors secreted by *S. scabies* and is a potent inhibitor of cellulose synthesis (Bischoff *et al.*, 2009; Scheible *et al.*, 2003). Protein secretion is also essential for the pathogenesis of *S. scabies*. Nec1, which is probably secreted by the Sec pathway, is a protein that, to date, has only been found in pathogenic streptomycetes, and is required for root colonization (Bukhalid and Loria, 1997; Joshi *et al.*, 2007a). The analysis of protein secretion by the *S. scabies* Tat pathway has shown that at least seven Tat substrates also contribute to virulence (Joshi *et al.*, 2010), and there are likely to be additional proteinaceous virulence factors yet to be identified.

In this study, we have characterized the T7SS of *S. scabies*. Surprisingly, we show that, unlike most T7SSs previously characterized in pathogenic bacteria, the *S. scabies* T7S machinery is fully dispensable for virulence. However, as reported previously for one of the T7SSs of the nonpathogen *Streptomyces coelicolor*, disruption of the genes encoding the EsxA and EsxB proteins results in delayed sporulation and abnormal spore phenotypes (Akpe San Roman *et al.*, 2010). We further show that strains lacking *esxA* or *esxB* are resistant to lysis by the *Streptomyces*-specific phage ϕ C31. Interestingly, the delayed sporulation and phage resistance phenotypes are unique to strains deleted for one or other of *esxA* or *esxB*, and are not observed when other essential components of the T7SS are lacking. Taken together, these results imply an unexpected intracellular role for EsxA and EsxB.

RESULTS

Genes coding for conserved components of a T7SS are present in *S. scabies*

To determine whether a T7SS was encoded in the genome of *S. scabies* (accession number FN554889), BLASTP analysis was performed using the amino acid sequences of components of the previously analysed T7SS from *S. coelicolor* and *M. tuberculosis* (Akpe San Roman *et al.*, 2010; Bitter *et al.*, 2009). This led to the identification of a gene, *scab58621*, coding for the conserved EccC component (the FtsK-SpoIIIE domain protein), which was found to be in close proximity to a second gene, *scab58651*, which encodes the conserved EccD component (Fig. 1A). EccD is a highly hydrophobic protein, and analysis of the coding sequence of *S. scabies* EccD suggests that it has 11 transmembrane domains.

A hallmark of a T7SS is the presence of genes coding for EsxA and EsxB, small proteins of the WXG100 protein family that usually contain a conserved W-x-G motif. Simple BLASTP analysis using the *S. coelicolor* WXG100 protein sequences EsxA (Sco5725), EsxB (Sco5724) and Sco4509 (Akpe San Roman *et al.*, 2010; Palmer and Hutchings, 2010) to query the *S. scabies* protein database failed to identify any obvious homologues. However,

visual inspection of the genomic region around *scab58621* revealed the presence of three open reading frames (ORFs) (*scab58661*, *scab58671* and *scab58681*) encoding proteins of the expected size for a typical WXG100 protein (100–150 amino acids). The translated protein sequences were analysed using the secondary structure prediction program PHYRE (Kelley and Sternberg, 2009), and both SCAB58671 (henceforth EsxA) and SCAB58681 (EsxB) were predicted to be members of the EsxAB dimer-like superfamily of proteins, with estimated precision values of 95% (*E*-value 0.02) and 85% (*E*-value 0.34), respectively. Neither EsxA nor EsxB contains the canonical WXG motif, with EsxB having an -FNG- motif at this position and EsxA having an -FQA- motif (Fig. S1, see Supporting Information).

Other conserved T7SS components found in *M. tuberculosis* and *S. coelicolor* are EccB (Sco5721 in *S. coelicolor*), a predicted monotopic membrane protein, and one or more subtilisin-like serine proteases (MycP1–5 in *M. tuberculosis*, Sco5722 and Sco5731 in *S. coelicolor*). The *S. scabies* genome does not encode any protein with similarity to EccB, but does encode several predicted subtilisin-type serine proteases, although none of them are encoded in the proximity of the T7SS. Of the other genes that are located close to SCAB58621, SCAB58631 is a proline/alanine-rich protein that is homologous to *M. tuberculosis* EspI (BLAST *E*-value of $7e^{-13}$ in 231 amino acid overlap) and SCAB58611 is a conserved hypothetical protein, the closest homologue of which in *M. tuberculosis* is the PPE family protein Rv2352c (BLAST *E*-value of 0.019 in 109 amino acid overlap). SCAB58661 is a predicted monotopic membrane protein, whose closest orthologues are encoded in some *Streptomyces* and also appear to be linked to putative T7SS gene clusters. However, no closely related proteins to SCAB58661 are encoded in any other genus. The genetic organization of the *S. scabies* T7SS is shown in Fig. 1A.

The *esxA* and *esxB* genes form a transcription unit and are expressed under laboratory growth conditions

The genetic organization and spacing are consistent with the genes *scab58681* (*esxA*) to *scab58611* being organized as a probable transcription unit. To ascertain whether this probable transcription unit was expressed, we isolated mRNA and prepared complementary DNA (cDNA) from the *S. scabies* 87.22 wild-type strain that had been cultured for 20 h in either tryptone soy broth medium (a standard laboratory growth medium for *Streptomyces*; Kieser *et al.*, 2000) or thaxtomin defined medium (which contains cellobiose and is known to induce the expression of *S. scabies* virulence genes, e.g. Bignell *et al.*, 2010; Johnson *et al.*, 2007; Joshi *et al.*, 2007a). The cDNA was used as a template in polymerase chain reactions (PCRs) to determine whether the individual *esxA* (*scab58681*) and *esxB* (*scab58671*) genes were expressed in either of these growth conditions, and whether they were co-transcribed with each other, and with the neighbouring *scab58661* gene.

Fig. 1 Genetic organization of the type VII secretion system (T7SS) coding region in *Streptomyces scabiei*. (A) Organization of the genes coding for T7SS components on the chromosome of *S. scabiei*. The numbers show the sizes of the intergenic regions between each gene, in base pairs (bp). (B) The *esxA* and *esxB* genes are co-expressed. RNA was isolated from *S. scabiei* cultured on tryptone soy broth (TSB) or thaxtomin defined growth medium (TDM) and cDNA was prepared as described in Experimental procedures. cDNA was used as a template in polymerase chain reactions (PCRs) using primers that amplify within *esxA* or *esxB*, or across the *esxA-esxB* or *esxB-scab58661* junctions. The expected sizes of the amplified products are indicated to the right. The RNA sample without the reverse transcriptase step served as a template for a negative control and yielded no products (and is therefore not shown). Chromosomal DNA was also used as a template with the same primers as a positive control. Samples were analysed on the same agarose gel; intervening lanes have been excised for clarity.

As shown in Fig. 1B, PCR products of the expected sizes were obtained for the *esxA* and *esxB* genes under both of the growth conditions tested. No products were obtained if the reverse transcriptase step was omitted, indicating that the mRNA was not contaminated with chromosomal DNA (not shown). Furthermore, amplified DNA products of the expected sizes were also seen when oligonucleotide primers were used that span the *esxA* and *esxB* genes, and the *esxB-scab58661* genes. This indicates that *esxA* and *esxB* are expressed under standard laboratory growth conditions and that the two genes form a transcription unit together with at least the adjacent downstream gene, *scab58661*.

Mutations in genes coding for core T7SS components have differential effects on *S. scabiei* growth and development

In order to investigate the requirement of the *S. scabiei* T7SS in its physiology, and to explore any role in virulence, we constructed

gene deletions in the *esxA*, *esxB*, *eccC* and *eccD* genes, that were each marked with an apramycin resistance cassette, using the λ RED REDIRECT approach (Gust *et al.*, 2003). Each mutant strain was verified by Southern blot analysis (Fig. S2, see Supporting Information), and the verified strains were designated JFK1 ($\Delta eccC::Apra$), JFK2 ($\Delta eccD::Apra$), JFK3 ($\Delta esxB::Apra$) and JFK4 ($\Delta esxA::Apra$).

We firstly investigated whether any of these mutations affected the growth of *S. scabiei* by assessing the ability of strains JFK1–JFK4 to grow on nine different types of solid media typically used for the laboratory cultivation of *Streptomyces* strains (Fig. 2). Clear differences in strain behaviour were revealed by this analysis on some of the growth media. For example, the strain carrying an apramycin-marked deletion of *eccC* (JFK1) appeared to form aerial hyphae (observed as a white appearance to the surface of the biomass) more readily than any of the other strains, including the wild-type strain, on R2 medium or R2 medium containing yeast extract (Fig. 2D,E). Similarly, when grown on soyflour mannitol

Fig. 2 Growth analysis of *Streptomyces scabies* strains carrying mutations in the *esxA*, *esxB*, *eccC* and *eccD* genes. (A–I) Phenotypic analysis of growth on solid media. Equal numbers of spores of strain 87.22 (wild type, WT), JKF1 (*eccC*⁻), JKF2 (*eccD*⁻), JKF3 (*esxB*⁻) and JKF4 (*esxA*⁻) strains were streaked from glycerol stock and incubated at 30 °C for 5 days. The different growth media used were soyflour mannitol (A), oatbran (B), instant potato mash (C), R2 (D), R2 with yeast extract (E), minimal with 1% mannitol (F), minimal with 1% glucose (G), Difco nutrient agar (H) and yeast malt extract (I). The images shown are representative examples from five independent growth experiments. (J) Growth rate analysis of the same strains in tryptone soy broth. Aliquots of tryptone soy broth (100 mL) were inoculated with spores of the strain of interest to a density of 1×10^6 spores/mL of culture medium, and incubated at 30 °C with shaking. Samples (1 mL) were withdrawn from the cultures every 3 h and total cytosolic protein was prepared as described in Experimental procedures. Error bars represent the standard error of the mean, where $n = 3$.

medium (Fig. 2A), the same strain also showed increased sporulation in comparison with the wild-type (observed as darker grey pigmentation). These observations indicate that the loss of *eccC*, which encodes the FtsK-SpoIIIE domain protein, results in a media-dependent acceleration of the *S. scabies* developmental cycle.

By contrast, strains carrying marked deletions of *esxA* (JKF4) or *esxB* (JKF3) showed a decrease in aerial hyphae formation and sporulation in comparison with the wild-type strain on several different growth media, including soyflour mannitol medium (Fig. 2A) oatbran medium (Fig. 2B), instant potato mash medium (Fig. 2C), minimal medium plus glucose (Fig. 2G) and, most prominently, yeast malt extract (Fig. 2I). In each case, it appeared that the growth defect was slightly more pronounced for JKF4 than for

JKF3. Thus, it appears that the inactivation of genes coding for the EsxAB dimer-like superfamily proteins results in a developmental delay. A similar developmental delay has been reported for a strain of *S. coelicolor* with a transposon insertion in *esxB* (Akpe San Roman *et al.*, 2010). It should be noted that strain JKF2 (which carries a marked deletion of *eccD*) showed a similar growth behaviour to the wild-type on all the solid growth media tested.

We next assessed the growth of the strains in tryptone soybroth liquid culture. *Streptomyces scabies*, like *S. coelicolor*, does not sporulate in liquid culture, and so these experiments primarily assess vegetative growth. As *Streptomyces* strains grow as mycelial clumps in liquid culture, we assessed growth by harvesting mycelia at different time points and by measuring total cytosolic

Fig. 3 Scanning electron microscopy images of spore chains from the following *Streptomyces scabiei* strains: (A, F) 87.22 (wild-type); (B, G) JKF1 (*eccC*⁻); (C, H) JKF2 (*eccD*⁻); (D, I) JKF3 (*esxB*⁻); (E, J) JKF4 (*esxA*⁻). The strains were inoculated onto instant potato mash agar from glycerol stock and incubated at 30 °C for 10 days.

protein (Joshi *et al.*, 2010; Widdick *et al.*, 2006; Fig. 2J). Strains JKF1 and JKF2 grew similarly to the wild-type strain, entering the logarithmic growth phase between 21 and 24 h post-inoculation and reaching a peak by 33 h post-inoculation. By contrast, strains JKF3 and JKF4 entered the logarithmic growth phase earlier than the wild-type strain, at 18 h post-inoculation. The rate of growth remained similar to that of the other strains for the duration of the logarithmic growth period, as each slope had a similar gradient (Fig. 2J). Strain JKF4 entered the stationary phase at 27 h post-inoculation, whereas all of the other strains, including the wild-type, entered the stationary phase at 33 h post-inoculation. However, the final levels of total cytosolic protein were similar for all of the strains, indicating that similar biomasses were achieved at the end of the growth cycle.

Taken together these results suggest that loss of *esxA* and *esxB* affects normal growth and development of *S. scabiei*, resulting in growth acceleration during the early growth phase, but a clear delay in the onset of aerial hyphae production and sporulation. Interestingly, however, loss of the gene coding for a key component of the T7SS, the FtsK-SpoIIIE domain protein EccC did not phenocopy the *esxA* and *esxB* strains and had noticeably different effects on *S. scabiei* development than the loss of EsxA and EsxB.

Spore chains of strains lacking EsxA and EsxB show morphological defects

As we observed delayed sporulation of *S. scabiei* strains carrying marked mutations in *esxA* or *esxB*, we next analysed the morphology of the spore chains of *S. scabiei* that had been cultured on instant potato mash agar. This was chosen as it permits the sporu-

lation of the *esxA* and *esxB* mutant strains and is the medium in which the development of these strains most closely resembles that of the wild-type (Fig. 2C). The *S. scabiei* wild-type strain, together with JKF1–JKF4, were cultured on instant potato mash agar, and samples of each strain showing spore development were prepared following 10 days of growth for analysis by scanning electron microscopy.

As shown in Fig. 3, abundant spirally arranged spore chains were visible for the wild-type strain and for the strains carrying marked deletions in *eccC* or *eccD* (Fig. 3A–C,F,G). However, for the strains carrying marked deletions in *esxA* or *esxB*, there was very little evidence of spiral spore chain formation (Fig. 3D,E,I,J). When examined under higher magnification, it could also be seen that the pre-spores in the spore chains from strains JKF3 and JKF4 were not of uniform size, with many lysed aerial hyphae and irregularly sized pre-spores (compare Fig. 3I,J with Fig. 3F–H). Thus, it seems that EsxA and EsxB (but not EccC or EccD) are not only required for the onset of aerial hyphae formation and sporulation (at least on some growth media), but also for correct spore formation.

Introduction of a second copy of *esxA*–*esxB* into wild-type *S. scabiei* alters the timing of development

To attempt to complement the sporulation defect of strains JKF3 and JKF4, we constructed an integrating vector carrying *esxA* and *esxB*, together with 225 base pairs upstream of *esxA*, which should cover the natural promoter region (predicted to lie approximately 100 base pairs upstream of the *esxA* start codon according to the BPROM prediction program; <http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb>).

Fig. 4 Partial complementation of the developmental defect of the *Streptomyces scabies* *esxA* and *esxB* strains is afforded by supplying an *in trans* copy of *esxAB*. Equal numbers of spores of strains 87.22 (wild-type, WT), 87.22/*pesxAB*, JKF3 (*esxB*⁻), JKF3/*pesxAB*, JKF4 (*esxA*⁻) and JKF4/*pesxAB* were streaked from glycerol stock onto yeast malt extract medium and incubated at 30 °C for the indicated time periods.

We introduced this construct into the marked *esxA* and *esxB* deletion strains, and also into the wild-type strain, and cultured these, alongside the nontransformed strains, on yeast malt extract agar. This was chosen as growth medium as it gave the most prominent developmental defect for the *esxA* and *esxB* deletion strains (Fig. 2I), and was therefore expected to most clearly indicate whether complementation of these strains was successful.

Following 2 days of growth on this medium, almost the entire surface of the biomass formed by the wild-type strain was covered with white aerial hyphae (Fig. 4). By contrast, the development of aerial hyphae appeared to be delayed for the wild-type strain harbouring an additional copy of *esxAB*, as it was only evident at the edges of the biomass. As expected, no aerial hyphae were visible for the *esxA* or *esxB* marked deletion strains; however, the same strains carrying an ectopic copy of *esxAB* had started to show some formation of aerial hyphae around the edges of the biomass.

After 6 days of growth, the wild-type strain had sporulated (observed as a grey appearance on the surface of the biomass in Fig. 4). Sporulation was also observed for the wild-type strain harbouring an extra copy of *esxAB*, but, in this case, the surface of the biomass appeared darker than that of the untransformed wild-type strain, indicating that more spores were produced following the introduction of a second copy of *esxA* and *esxB*. Both of the *esxA* and *esxB* mutant strains showed more prolific formation of aerial hyphae when they carried an ectopic copy of *esxAB*, although development was still clearly retarded relative to the wild-type strain. This indicates that partial complementation of the mutant strains was afforded by supplying an additional copy of *esxA* and *esxB* *in trans*.

Taken together, these results indicate that the cellular levels of EsxA and EsxB (and possibly the relative amounts of the two proteins) are critical for the timing of *S. scabies* development.

Loss of EsxA and EsxB confers resistance to infection by bacteriophage ϕ C31

As T7SS components have been associated with bacteriophage infection, we carried out plaque assays using two different *Streptomyces*-specific bacteriophages, ϕ R4 and ϕ C31, which are in

different immune groups (Chater and Carter, 1979). Each of the bacteriophages tested was able to form plaques on the *S. scabies* wild-type strain, with ϕ R4 producing very small plaques and ϕ C31 very large plaques (Fig. 5). Each of the T7S mutant strains was also susceptible to lysis by ϕ R4, giving similar numbers and similar plaque sizes to those seen during infection of the wild-type strain (Fig. 5, top panel). Interestingly, although the strains carrying marked mutations in *eccC* or *eccD* behaved similarly to the wild-type strain for ϕ C31 infection, the strains with marked deletions in *esxA* or *esxB* appeared to be fully resistant to infection, as there was a complete absence of plaque formation (Fig. 5, bottom panel). This implies that the EsxAB dimer-like superfamily proteins are required for lytic infection by ϕ C31, but not by ϕ R4.

The T7SS is dispensable for *S. scabies* virulence

Streptomyces scabies is a broad host range pathogen that causes root rot on both model plant species and agricultural crops (reviewed in Loria *et al.*, 2006). We used three different plant hosts and bioassays to assess virulence, namely the ability to cause necrosis *in vitro* on disc slices of potato tuber (Fig. 6A), and to cause necrosis on, and affect the growth of, radish (Fig. 6B) and tobacco (Fig. 7).

The first virulence bioassay performed involved the inoculation of aseptically prepared potato tuber slices with plugs taken from agar plates showing confluent sporulation of each strain. Lesion size and severity were assessed following 7 days of incubation. In this potato disc assay (Fig. 6A), it was not possible to differentiate the size and severity of lesions among tuber discs inoculated with the wild-type strain and the T7SS mutant strains (JKF1–JKF4). In contrast, the noninoculated tuber disc lacked the necrosis and pitting found in the inoculated tuber tissue.

The second virulence bioassay performed involved the inoculation of young radish seedlings with an equal number of spores of each strain. Disease symptoms were assessed following 6 days of incubation. The root growth of radish seedlings inoculated with the wild-type strain (Fig. 6B) was severely stunted when compared with that of water-inoculated seedlings (marked 'control' in Fig. 6B), and there was browning at both the root tip and at regions in which the formation of secondary roots would have

Fig. 5 The *Streptomyces scabies* *esxA* and *esxB* mutant strains are resistant to infection by bacteriophage ϕ C31. Approximately 10^8 spores of *S. scabies* strains 87.22 (wild-type, WT), JKF1 (*eccC*⁻), JKF2 (*eccD*⁻), JKF3 (*esxB*⁻) and JKF4 (*esxA*⁻) were mixed with a suspension of bacteriophage ϕ R4 (top panel) or ϕ C31 (bottom panel). Plates were incubated at 30 °C for 18 h to allow plaque formation prior to being photographed.

Fig. 6 The *Streptomyces scabies* *eccC*⁻, *eccD*⁻, *esxB*⁻ and *esxA*⁻ strains show necrosis on potato tuber slices and are virulent for infection in the radish root assay. (A) Potato tuber slices in Petri dishes were inoculated spore side down with agar plugs from plates of each of the indicated strains showing confluent sporulation, as described in Experimental procedures. The Petri dishes were sealed with parafilm and incubated in the dark at room temperature for 7 days prior to being photographed. (B) Radish seeds, which had been grown as described in Experimental procedures, were transferred to water agar and inoculated with equal numbers of spores of each of the indicated *S. scabies* strains. Petri dishes were sealed with parafilm and incubated at 21 ± 2 °C with a 16-h photoperiod for 6 days prior to the excision of roots from the agar. Seedlings were assessed for disease symptoms and photographed. Scale bars, 1 cm.

occurred in noninoculated plants. However, similar symptoms were observed when radish seedlings were inoculated with each of the JKF1–JKF4 strains.

The third virulence bioassay performed involved the inoculation of 7-day-old *Nicotiana tabacum* seedlings growing in agar with an equal number of spores of each strain. Disease symptoms were assessed following 9 weeks of incubation. The growth of seedlings inoculated with the wild-type strain was severely reduced com-

pared with that of water-inoculated control seedlings (Fig. 7). The plant height was greatly reduced (Fig. 7A), although the overall condition of the plants appeared healthy, with little etiolation of the stems and leaves (indeed, when viewed from above in Fig. 7B, it is difficult to distinguish control plants from *S. scabies*-infected plants). This is consistent with reports that the foliage of potato plants infected with *S. scabies* usually shows no evidence of infection. Inspection of the roots revealed that they were

Fig. 7 The *Streptomyces scabies* *eccC*⁻, *eccD*⁻, *esxB*⁻ and *esxA*⁻ strains show virulence on *Nicotiana tabacum*. Tobacco seedlings, germinated in individual magenta boxes as described in Experimental procedures, were inoculated with approximately 5×10^7 spores of the indicated strains. Plants were incubated at 21 ± 2 °C with a 16-h photoperiod for 9 weeks prior to being photographed from the side (A), above (B) and below (C). Scale bars, 2 cm.

underdeveloped, possessing much less root mass (estimated visually), and there was very little evidence of lateral roots (Fig. 7C). However, broadly similar effects were observed when *Nicotiana* seedlings were inoculated with each of the *S. scabies* mutant strains JKF1–JKF4 (Fig. 7).

DISCUSSION

Protein secretion systems are intimately linked to virulence in all bacterial pathogens (e.g. Alvarez-Martinez and Christie, 2009;

Lindeberg *et al.*, 2009), and even those protein export systems that play a housekeeping role in some organisms are frequently co-opted for virulence functions during pathogenesis (e.g. Joshi *et al.*, 2010; Rigel and Braunstein, 2008). In this study, we have addressed the role of the putative T7SS encoded in the genome of *S. scabies* in relation to its physiology and virulence. We constructed marked mutations in genes coding for two of the components, EccC and EccD, that have previously been reported to be essential for T7S in *M. tuberculosis* (Brodin *et al.*, 2006; Raghavan *et al.*, 2008; Stanley *et al.*, 2003), as well as each of the two genes coding for the small secreted proteins, EsxA and EsxB.

Surprisingly, we found that there was no detectable role for any of the T7S components in *S. scabies* virulence in any of the plant infection models tested. This is in contrast with a clear role for this system in the virulence of the animal pathogens *M. tuberculosis*, *M. bovis*, *S. aureus* and *B. anthracis* (Burts *et al.*, 2005; Garufi *et al.*, 2008; Hsu *et al.*, 2003; Pym *et al.*, 2003; Stanley *et al.*, 2003). Studies with *Listeria monocytogenes* showed that a deletion of the gene coding for the ESAT-6 (EsxB) homologue did not affect virulence in a mouse model of infection, although any role for the secretion system itself in this study was not investigated (Way and Wilson, 2005). It remains possible that the T7SS of *S. scabies* plays a subtle role during infection that we cannot reliably detect in the qualitative growth tests used here to assess virulence.

In contrast with the apparent lack of any role for the T7SS in plant infection, we clearly demonstrated that components encoded by the T7SS gene cluster are required for the normal growth and development of *S. scabies*. This is in close agreement with the studies of Akpe San Roman *et al.* (2010) in the related streptomycete *S. coelicolor*. We showed that the loss of the genes coding for EsxA and EsxB leads to a failure of *S. scabies* to undergo normal aerial development and sporulation during the growth cycle on several types of solid growth media. Moreover, any spore chains that were formed by these mutant strains were clearly aberrant with irregularly sized pre-spores. This is very similar to the findings of Akpe San Roman *et al.* (2010), who showed that the disruption of *esxB* resulted in a sporulation delay, and that the spore morphology and DNA content were substantially affected in the knockout strain.

It is intriguing to note that phenotypic differences have been observed previously between strains lacking EccC, an essential component of the T7S secretion apparatus, and strains lacking EsxA and EsxB, the substrate proteins of the T7SS. Wherever examined, including *M. tuberculosis*, *M. smegmatis*, *S. aureus* and *S. coelicolor*, loss of EccC abolishes the export of EsxA and EsxB (Akpe San Roman *et al.*, 2010; Burts *et al.*, 2005; Coros *et al.*, 2008; Stanley *et al.*, 2003). In both *S. scabies* and *S. coelicolor*, the *eccC* mutant strain does not phenocopy the *esxA/esxB* strains. This indicates that it is not simply the case that failure to secrete EsxA and EsxB gives rise to the aberrant development of *Streptomyces*.

Instead, it points to an intracellular role for these two small proteins. This is also supported by our findings that an ectopic copy of *esxAB* expressed in an otherwise wild-type *S. scabies* strain also interferes with the developmental cycle, as this might be expected to shift the balance between intracellular and extracellular EsxA and EsxB in this situation.

It has been proposed that the EsxAB heterodimer controls the co-ordination of cell division with the segregation of nucleoids, most probably through interaction with other factors (for example, the morphogenic regulator BldB) that regulate nucleoid condensation (Akpe San Roman *et al.*, 2010). Although BldB is directly linked to the T7S-encoding loci in some streptomycetes, the *S. scabies* BldB homologue, encoded by *scab25271*, does not cluster with this locus. It is interesting to note that the T7SS of the saprophyte *M. smegmatis* is essential for conjugal DNA transfer, with the implication that either co-secretion of EsxAB with other T7S substrate proteins, and/or EsxAB interaction with other proteins in the cell envelopes of the donor and recipient, mediates DNA transfer (Coros *et al.*, 2008; Flint *et al.*, 2004).

One of the components encoded by the putative T7SS of *Bacillus subtilis*, YueB, is a membrane protein which is essential for irreversible binding of the bacteriophage SPP1 to the bacterial surface and ejection of phage DNA into the host cell (Sao-Jose *et al.*, 2004, 2006). Furthermore, it has been shown that, although *yukA* and *yukE* (coding for an EccC homologue and an EsxA homologue, respectively) are not essential for phage infection, strains with mutations in these genes give smaller plaques (Sao-Jose *et al.*, 2004).

In this study, we have shown that the loss of EsxA and EsxB prevents lytic infection by the bacteriophage ϕ C31, another process which is also closely linked with DNA mobility. However, a strain lacking the FtsK-SpoIIIE domain protein, EccC, which is essential for the export of EsxA and EsxB to the cell envelope in all organisms examined to date (Akpe San Roman *et al.*, 2010; Burts *et al.*, 2005; Champion *et al.*, 2006), behaves very differently to the *esxA/esxB* deletion strains by showing sensitivity to ϕ C31 infection. Like the sporulation defect, this phenotype appears to be linked to intracellular levels of EsxA/B.

It is possible that intracellular EsxA and EsxB interact with proteins that regulate phage DNA integration and/or excision or, alternatively, it may be that EsxA/B themselves interact with DNA. Regardless of the precise intracellular roles for EsxA and EsxB, one of the major functions of the T7S machinery of *Streptomyces*, identified by Akpe San Roman *et al.* (2010) and confirmed here, is the regulated secretion of EsxA and EsxB to control the progression of the developmental growth cycle. Other roles for the T7SS or extracellular EsxA and EsxB cannot be ruled out; however, they are likely to be subtle in comparison with the intracellular role identified here.

In conclusion, we have shown that two small proteins encoded at the T7SS locus of *S. scabies* are required for normal growth of

the organism, and that the probable role of the T7SS is in the regulated secretion of these proteins. Although we observed no overt virulence phenotype associated with the loss of T7SS components or substrates in *S. scabies*, it should be noted that, for the most part, the virulence assays used here are associated with the growth of vegetative hyphae (e.g. Joshi *et al.*, 2010). However, dispersal of the pathogen through the environment is mediated by spores, and therefore any mutation that leads to a defect in sporulation would be expected to have a major effect on the dissemination of *S. scabies* during infection.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids, media and culture conditions

Streptomyces scabies strains used in this study were routinely cultured on instant potato mash agar (Joshi *et al.*, 2010) and incubated at 30 °C. Where required, the medium was supplemented with apramycin (100 µg/mL), hygromycin B (100 µg/mL), kanamycin (50 µg/mL) or nalidixic acid (25 µg/mL). Spore stocks were prepared as described previously (Kieser *et al.*, 2000).

Targeted disruption of the *S. scabies* *eccC*, *eccD*, *esxB* and *esxA* genes employed the REDIRECT PCR targeting system (Gust *et al.*, 2003), with *Escherichia coli* strain BW25113 containing pIJ790 (Gust *et al.*, 2003) and *S. scabies* cosmid 212 (which harbours all of the genes of the T7SS-encoding cluster) as the initial host. Plasmid pIJ773 served as the source of the apramycin resistance cassette, which was amplified using the oligonucleotide primer pairs listed in Table S1 (see Supporting Information) designed to disrupt the individual genes of interest. After individual disruption of each gene, mutant cosmids were conjugated into *S. scabies* from the *E. coli* methylation-deficient host strain ET12567/pUZ8002 (Bignell *et al.*, 2010; MacNeil *et al.*, 1992). Double crossovers were selected by resistance to apramycin and sensitivity to kanamycin. Gene deletions were verified by Southern blot analysis using the method of Bignell *et al.* (2010). For this, *S. scabies* strains were cultured in tryptone soy broth (Kieser *et al.*, 2000) and incubated at 30 °C with shaking at 200 rpm for 2 days prior to extraction of chromosomal DNA using the MasterPure Gram-positive DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA). The oligonucleotides used for probe preparation are given in Table S1. The verified strains were designated JKF1 (Δ *eccC*::Apra), JKF2 (Δ *eccD*::Apra), JKF3 (Δ *esxB*::Apra) and JKF4 (Δ *esxA*::Apra).

For the ectopic expression of *esxAB*, DNA covering the *esxA* and *esxB* region, together with 225 bp of upstream DNA and 93 bp of downstream DNA, was amplified from cosmid 212 using oligonucleotide primers *esxA-B_comp_fwd* and *esxAB_comp_rev* (Table S1). Following digestion with *EcoRI* and *EcoRV*, it was ligated into similarly digested pBluescript, verified by DNA sequencing and subcloned into pSET-Hyg (Muller *et al.*, 2007) as an *EcoRI*–*EcoRV* fragment, yielding *pesxAB*. This plasmid was then transferred to *S. scabies* by intergeneric conjugation, after which integration into the chromosome at the ϕ C31 attachment site was selected by hygromycin resistance.

For growth rate analysis, 100 mL of tryptone soy broth (Kieser *et al.*, 2000) was inoculated with *S. scabies* spores to a final spore concentration

of approximately 1×10^6 spores/mL; 1-mL samples, in triplicate, were removed every 3 h over the course of 45 h and mycelia were pelleted by centrifugation. Following resuspension in 1 mL of 1 M NaOH, samples were boiled for 10 min and the supernatant obtained following centrifugation was used to estimate the total cytosolic protein concentration in the sample, employing the Bio-Rad DC protein assay kit (Hemel Hempstead, Hertfordshire, UK).

For phenotypic analysis, *S. scabiei* strains were cultured alongside each other on soy flour mannitol agar (Hobbs *et al.*, 1989), oat bran agar (4% oat bran and 1.2% agar in tap water), instant potato mash agar, R2 (Okanishi *et al.*, 1974), R2YE (as R2 with 0.5% yeast extract), minimal media (Hopwood, 1967) supplemented with either 1% mannitol or 1% glucose, Difco nutrient agar (Kieser *et al.*, 2000) or yeast malt extract agar (YME; 0.4% glucose, 0.4% yeast extract, 1% malt extract and 1.5% agar in tap water).

For the detection of *esxA* and *esxB* transcripts, *S. scabiei* was cultured in either tryptone soy broth or thaxtomin defined medium (Johnson *et al.*, 2007) at 30 °C with shaking for 20 h, after which time RNA was isolated as described previously (Joshi *et al.*, 2007b). An additional incubation step with DNase (Qiagen, Valencia, CA, USA) at 37 °C for 30 min was included to ensure the destruction of genomic DNA, which was subsequently removed using StrataClean Resin (Stratagene, San Diego, CA, USA). Synthesis of cDNA from RNA (2 ng) was performed with random hexamer primers using a SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Control reactions were carried out that excluded reverse transcriptase from the reaction mix (NRT sample). Transcript analysis using cDNA as PCR template used oligonucleotide primer pairs *esxA*_transcript_fwd and *esxA*_transcript_rev (to detect *esxA* expression), *esxB*_transcript_fwd and *esxB*_transcript_rev (to detect *esxB* expression), *esxA*_transcript_fwd and *esxA*_transcript_rev (to detect *esxA*–*esxB* co-expression) or *esxB*-ssc58661_fwd and *esxB*-ssc58661_rev (to detect *esxB*–*scab58661* co-expression) (sequences listed in Table S1). The absence of genomic DNA was confirmed as no PCR products were obtained when NRT samples were used as the template.

Scanning electron microscopy of *S. scabiei* spore chains

Spores of *S. scabiei* strains 87-22, JFK1, JFK2, JFK3 or JFK4 were inoculated onto instant potato mash agar and incubated at 30 °C for 10 days to allow confluent sporulation. A square section was excised from the agar surface of each plate and mounted on the sample holder using Tissue-Tech OCT Compound (BDH Laboratory Supplies, Poole, UK). This was rapidly frozen in liquid N₂ slush and then transferred under vacuum to the cryopreparation stage and warmed to –95 °C for 5 min to remove surface water. After sublimation, the samples were then cooled to –115 °C and coated with approximately 5 nm of Au/Pd. Samples were examined using a Philips XL 30 emission scanning electron microscope (ESEM) operating at an accelerating voltage of 15 kV.

Phage plaque assays

Phage plaque assays were performed as described previously (Dowding, 1973). Briefly, serial dilutions of phage were prepared in Difco nutrient broth and 100-μL aliquots were transferred to the surface of Difco nutrient

agar supplemented with 0.5% glucose, 10 mM MgSO₄ and an appropriate concentration of calcium ions (8 mM for φC31 and 25 mM for φR4). Spores of *S. scabiei* (to a final concentration of approximately 10^7 – 10^8 /mL) were added to molten soft nutrient agar; 1-mL aliquots were then transferred to the surface of the media containing the phage, allowed to set for 15 min, inverted and incubated at 30 °C for 18–20 h, after which time plaque formation was assessed.

Virulence bioassays

Potato tuber slice assay

Potato tuber discs (cv. Chippewa) were prepared, surface sterilized as described previously (Loria *et al.*, 1995) and transferred to a Petri dish lined with moist filter paper. Agar plugs excised from instant potato mash agar plates of *S. scabiei* strains showing confluent sporulation were placed spore side down onto the potato tissue, and each Petri dish was sealed with parafilm. As a control, an agar plug was also excised from a sterile plate and placed on the potato tissue. The discs were incubated in the dark at room temperature for 7 days.

Radish seedling assay

Radish seeds were surface sterilized [5 min in 70% (v/v) ethanol, followed by 10 min in 15% (v/v) bleach (Chlorox) and then several washes with sterile water) and then transferred to a moist lined Petri dish, sealed with parafilm and incubated in the dark at room temperature for 40 h to germinate. Seedlings were selected that displayed a similar level of germination, and were transferred to wells in 1.5% water agar plates. Freshly prepared *S. scabiei* spore suspensions were diluted in sterile water to give an optical density at 600 nm (OD₆₀₀) of 0.5, and 200 μL of this suspension was used to inoculate the radish seedlings. Petri dishes were sealed with parafilm and incubated at 21 ± 2 °C with a 16-h photoperiod for 6 days.

Tobacco assay

Nicotiana tabacum seeds were surface sterilized by exposure to chlorine gas inside a sealed chamber for 3 h; 1 mL of 0.1% phytagar (Sigma-Aldrich, St Louis, MO, USA) was then added to the seeds, which were stored at 4 °C in the dark for 2–5 days prior to sowing onto Murashige and Skoog medium supplemented with 1% sucrose and 0.8% agar in sterile magenta boxes. These were incubated at 21 ± 2 °C with a 16-h photoperiod for 7 days; the surface of the agar in each box was then flooded with approximately 1 mL of sterile water containing 5×10^7 spores from a pre-enumerated spore stock of *S. scabiei*. Plants were incubated at 21 ± 2 °C with a 16-h photoperiod, and disease progression was monitored over a period of 9 weeks.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Alignment of EsxA and EsxB proteins from Gram-positive bacteria. EsxA and EsxB proteins from *Mycobacterium tuberculosis*, *Staphylococcus aureus* and *Streptomyces coelicolor* were aligned with SCAB58671 (*Streptomyces scabies* EsxA) and SCAB58681 (*S. scabies* EsxB) using CLUSTALW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

Fig. S2 Southern blot analysis of *Streptomyces scabies* strains JKF1, JKF2, JKF3 and JKF4 to confirm the chromosomal deletion of genes at the type VII secretion system (T7SS) gene cluster. Chromosomal DNA and DNA probe preparation and Southern blotting were carried out as described in Experimental procedures. (A) *SalI*-digested DNA from chromosomal DNA from *S. scabies* 87.22 (wild-type, WT) and JKF1 ($\Delta eccC::Apra$) was probed with a DNA probe that hybridizes upstream of *eccC*; (B) *SalI*-digested chromosomal DNA from *S. scabies* 87.22 (WT) and JKF2 ($\Delta eccD::Apra$) was probed with a DNA probe that hybridizes upstream of *eccD*; (C) *SalI*-digested chromosomal DNA from *S. scabies* 87.22 (WT) and JKF3 ($\Delta esxB::Apra$) was probed with a DNA probe that hybridizes upstream of *esxB*; (D) *MluI*-digested chromosomal DNA from *S. scabies* 87.22 (WT) and JKF4 ($\Delta esxA::Apra$) was probed with a DNA probe that hybridizes upstream of *esxA*. The expected sizes of the hybridizing DNA bands for the wild-type and mutant strains are indicated in (E).

Table S1 Oligonucleotides used in this study. Restriction sites are underlined.