Molecular Microbiology (2010) 77(4), 943–957
doi:10.1111/j.1365-2958.2010.07261.x
First published online 30 June 2010

Investigating lipoprotein biogenesis and function in the model Gram-positive bacterium *Streptomyces coelicolor*

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Summary

Lipoproteins are a distinct class of bacterial membrane proteins that are translocated across the cytoplasmic membrane primarily by the Sec general secretory pathway and then lipidated on a conserved cysteine by the enzyme lipoprotein diacylglycerol transferase (Lgt). The signal peptide is cleaved by lipoprotein signal peptidase (Lsp) to leave the lipid-modified cysteine at the N-terminus of the mature lipoprotein. In all Gram-positive bacteria tested to date this pathway is non-essential and the lipid attaches the protein to the outer leaflet of the cytoplasmic membrane. Here we identify lipoproteins in the model Gram-positive bacterium *Streptomyces coelicolor* using bioinformatics coupled with proteomic and downstream analysis. We report that *Streptomyces* species translocate large numbers of lipoproteins out via the Tat (twin arginine translocase) pathway and we present evidence that lipoprotein biogenesis might be an essential pathway in *S. coelicolor*. This is the first analysis of lipoproteins and lipoprotein biogenesis in *Streptomyces* and provides the first evidence that lipoprotein biogenesis could be essential in a Gram-positive bacterium. This report also provides the first experimental evidence that Tat plays a major role in the translocation of lipoproteins in a specific bacterium.

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Introduction

Bacteria live in a variety of different environments and must sense and respond to a multitude of stresses in order to survive. Both Gram-positive and Gram-negative bacteria contain proteins localized to their membranes which can interact with, and sense, their environment. Lipoproteins are a distinct class of membrane-associated proteins that play a key role in signal transduction and in nutrient scavenging and uptake in Gram-positive bacteria, acting as substrate binding proteins for a vast array of different ATP binding cassette (ABC) transporters (Bertram *et al.*, 2004; Hutchings *et al.*, 2006a). They are also involved in essential extracytoplasmic processes, including cell envelope biogenesis and protein folding (Hutchings *et al.*, 2009). It has been proposed that in Gram-positive bacteria lipoproteins are the equivalent of periplasmic proteins in Gram-negative bacteria, precisely because of their roles as substrate binding proteins (Nielsen and Lampen, 1982). The diacylglyceride lipid anchor prevents the proteins from being lost from the cell and also bypasses the problem of membrane destabilization that would likely result from the insertion of many more transmembrane helices. The lipidation step is tightly controlled as part of a multi-step reaction that occurs after translocation across the cytoplasmic membrane, and this pathway is unique to bacteria.

Bacterial lipoproteins can be identified by a characteristic signal peptide that directs them for export and contains a 'lipobox' sequence motif that is essential for correct lipoprotein processing. The lipobox motif is typically L_{-3} – $(A/S/T)_{-2}$ – $(G/A)_{-1}$ – C_{+1}, in which the +1 cysteine residue is invariant (Rahman *et al.*, 2008). Until recently it was thought that all bacterial lipoproteins were translocated via Sec, the general secretory pathway, although there have been a few recent reports describing lipoproteins that are translocated by the twin arginine translocase (Tat), a pathway usually reserved for fully folded proteins (Berks *et al.*, 2000; Gralnick *et al.*, 2006; Valente *et al.*, 2007). Following translocation, the enzyme lipoprotein diacylglycerol transferase (Lgt) adds a lipid molecule via a thioether

linkage to the sulphydryl group of the +1 cysteine in the lipobox. A dedicated lipoprotein signal peptidase (Lsp) then cleaves the signal peptide leaving the modified cysteine residue at the N-terminus (Hutchings *et al*., 2009) In Gram-positive bacteria the lipoproteins remain tethered to the outer face of the cytoplasmic membrane by the lipid group. In Gram-negative bacteria further steps can occur including the addition of another fatty acid to the amino terminus of the +1 cysteine residue by the enzyme lipoprotein N-acyl transferase (Lnt) and transport of most lipoproteins to the outer membrane via the lipoprotein localization (Lol) pathway (Narita *et al*., 2004; Tokuda, 2009). Lnt homologues are also encoded by the genomes of some high GC Gram-positive bacteria (*Actinobacteria*) and have been shown recently to N-acylate lipoproteins in mycobacteria (Rezwan *et al*., 2007; Tschumi *et al*., 2009). Despite an absence of Lnt homologues in the genomes of low GC Gram-positive bacteria (*Firmicutes*), there is evidence that lipoproteins are N-acylated in *Staphylococcus aureus* (Kurokawa *et al*., 2009).

The lipoprotein biogenesis pathway is essential for viability in Gram-negative bacteria and occurs strictly in the order Lgt to Lsp to Lnt. Mutants lacking the lipoprotein biosynthetic enzymes are likely to aggregate improperly processed lipoproteins in their cytoplasmic membranes. Mislocalization of outer membrane lipoproteins is likely to have devastating consequences for Gram-negative bacteria as lipoproteins are central to the efficient functioning of three of the major pathways leading to outer membrane biogenesis (Robichon *et al*., 2005; Tokuda, 2009). In all Gram-positive bacteria tested to date the Lgt and Lsp enzymes have been found to be non-essential, despite the existence of essential lipoproteins (Leskelä *et al*., 1999). Indeed, the rigid order of enzyme activity seen in Gram-negative bacteria appears less stringent in some Gram-positives, with *Listeria monocytogenes* Lsp able to act on non-lipidated lipoproteins in a *Δlgt* strain leading to their mass release into the growth medium (Baumgärtner *et al*., 2006). Lsp was also able to process the MtuA lipoprotein in a *Δlgt* strain of *Streptococcus uberis* (Denham *et al*., 2009).

Streptomyces coelicolor is the model organism for the actinomycete genus *Streptomyces*, which are best known for their prodigious production of antibiotics (Li and Vederas, 2009). *S. coelicolor* is a soil bacterium with a complex, saprophytic lifecycle that enables it to survive and thrive in this harsh environment. *S. coelicolor* secretes an estimated 819 (10.5%) of its encoded proteins and a large number of these are hydrolases (e.g. proteases, cellulases) that break down complex organic molecules (Chater *et al*., 2009). An equally significant proportion of the proteome (7.8%) is taken up by proteins with putative transport functions, including a large number of ABC transporters involved in the uptake of the products generated by the hydrolases and other useful substrates from the environment (Bentley *et al*., 2002; Bertram *et al*., 2004). The accessory substrate binding proteins for these ABC transporters are typically putative lipoproteins and yet, despite the importance of these pathways to the survival of *Streptomyces* species, very little is known about the functions of lipoproteins, or lipoprotein biogenesis, in this important genus of bacteria. Intriguingly, the *S. coelicolor* genome is one of very few bacterial genomes to encode two putative Lgt paralogues.

Here we report the first comprehensive analysis of the lipoproteome and lipoprotein biogenesis pathway in *Streptomyces.* We report that lipoproteins make up ~2.7% of the proteome of *S. coelicolor* with approximately 23% of these lipoproteins predicted to be translocated across the cytoplasmic membrane by Tat. Our experimental analysis identified several Tat-dependent lipoproteins which, coupled with the bioinformatics analysis, suggests that Tat is a common pathway for lipoprotein export in *Streptomyces.* Removal of the Lgt homologues individually has no effect on lipoprotein processing, suggesting one Lgt enzyme can complement the other, but a double *lgt* mutant could not be isolated. Deletion of *lsp* resulted in secondary mutations in *S. coelicolor* that meant that the *Δlsp* mutant could not be fully complemented *cis* or *in trans* with *lsp*. Deletion of *lsp* resulted in the loss of lipoproteins from the cytoplasmic membrane, which in turn resulted in a pleiotropic phenotype. The *Δlsp* strain exhibited weaker growth, an altered cell envelope, smaller colony size and delayed sporulation compared with the wild-type. Taken together, these results provide evidence that blocking lipoprotein biogenesis is highly deleterious to the fitness of *S. coelicolor* and may even be essential for its viability. We conclude that deletion of *lsp* leads to a large-scale loss of lipoproteins from the membrane, at least one of which must have an essential function.

Results and discussion

Identifying putative lipoproteins

All *S. coelicolor* protein sequences with a cysteine residue in the first 50 amino acids were matched against the G + LPP pattern and a revised version, which allow for the recognition of typical and atypical Gram-positive lipoprotein signal sequences with long N-termini (Rahman *et al*., 2008). All predicted lipoprotein sequences were confirmed using a range of bioinformatic tools (see *Experimental procedures*). This analysis confidently identified 201 putative lipoproteins in *S. coelicolor*. Eight more were identified by alternative strategies (notably homology with other lipoproteins) and some, including CseA (Hutchings *et al*., 2006b), require reannotation to incorporate their signal peptides. An additional 14 sequences with unclear

Table 1. Lipoprotein functions in *Streptomyces coelicolor.*

Function	Numbers	%	Examples	References
Solute binding proteins	92	41	SCO5113 (BldK)	Nodwell *et al.* (1996)
Putative enzymes	36	16		
Redox processes	6	3	SCO4472 (ResA)	Lewin *et al.* (2008)
Signal transduction ('three component' systems)	6	3	SCO3011 (LpqB), SCO3357 (CseA)	Hoskisson and Hutchings (2006)
Cell envelope processes	7	3	SCO2153, SCO3194	
Function unknown	76	34		

signal peptide features were identified, suggesting up to 223 putative lipoproteins are present, accounting for ~2.7% of the *S. coelicolor* proteome (Table S1). Functional predictions (Tables S2 and S3) indicate that 40% of these lipoproteins are substrate binding proteins for ABC transporters (Table S2), for example BldK (Nodwell *et al.*, 1996), SCO2505 (ZurA) (Kallifidas *et al.*, 2010) and SCO2780 (DesE) (Barona-Gómez *et al.*, 2006), of which more than half are involved in carbohydrate uptake, reflecting the saprophytic lifestyle of *S. coelicolor*. Unusually, ~85% of the carbohydrate substrate binding proteins (notably those belonging to PFAM family PF01547; Table S2) are in loci that lack the requisite cytoplasmic ATP-binding proteins (Bertram *et al.*, 2004), suggesting that they are energized by ATP-binding protein(s) capable of interacting with multiple transport systems (Webb *et al.*, 2008). Candidates include the 'orphan' ATP binding proteins SCO4240 and SCO1707, which exhibit 92% and 51% amino acid identity, respectively, to MsiK of *Streptomyces reticuli*. MsiK energizes a cellobiose and maltose ABC transport system (Schlösser *et al.*, 1997) and can also interact with the DasABC system for N,N′-diacetylchitobiose transport (Saito *et al.*, 2008). Eight substrate binding proteins, including DesE, are genuine orphans and must interact with permease components encoded elsewhere in the *S. coelicolor* genome, consistent with recent reports on the diversity of ABC transporter organization (Thomas, 2009). Other putative lipoprotein functions include diverse enzymes, signal transduction (Hoskisson and Hutchings, 2006; Hutchings *et al.*, 2006b; Nguyen *et al.*, 2010), cell wall biosynthesis and homeostasis; redox processes including cytochrome *c* assembly (Worrall *et al.*, 2006; Lewin *et al.*, 2008) and protein folding (Table 1 and Table S3). Lipoproteins of unknown function make up 34% of the total and many of these are unique to members of the genus *Streptomyces* or to *S. coelicolor* specifically.

As several *S. coelicolor* putative lipoproteins have been shown to be Tat substrates (Table S1), the signal peptides of all the putative lipoproteins were inspected for Tat translocation motifs. This analysis indicated that 51 (23%) were putative Tat substrates. Thus, lipoproteins represent a significant proportion (*c.* 33%) of the approximately 150 Tat substrates predicted for *S. coelicolor*. Our detailed analysis is consistent with the recent suggestion that 28% of the putative lipoproteins of *S. coelicolor* are Tat substrates (Shruthi *et al.*, 2010).

Disrupting lipoprotein biogenesis

Lgt1 (SCO2034), Lgt2 (SCO7822) and Lsp (SCO2074) were identified by BLAST searching the *S. coelicolor* proteome with the corresponding sequences from *Bacillus subtilis, Escherichia coli* and *Mycobacterium tuberculosis* (Figs S1 and S2). *S. coelicolor lsp* was replaced with an apramycin resistance cassette to create an *lsp::apr* strain and the cassette was removed to create an unmarked *lsp* mutant. Deleting *lsp* proved difficult, with all resulting colonies exhibiting weak growth, suggesting loss of *lsp* is detrimental to the viability of *S. coelicolor.* Light and scanning electron microscopy revealed that the *Δlsp* strain forms small, flat colonies that are developmentally delayed (Fig. 1). The *lsp* mutant is also more sensitive than wild-type to lysozyme (MIC = 0.4 mg ml^{-1} versus 1.25 mg ml^{-1}), which targets the mature cell wall, and bacitracin (MIC = 35 μg ml^{-1} versus 100 μg ml^{-1}), which inhibits recycling of the lipid carrier during cell wall biosynthesis (Bouhss *et al.*, 2008). There was no difference in the sensitivity of the wild-type and *Δlsp* strains to the cell wall-specific antibiotic vancomycin, which targets cell wall precursors, or to beta-lactam antibiotics, which inhibit transpeptidase enzymes (commonly known as penicillin binding proteins, or PBPs) by binding to their active sites. Taken together, these data suggest that the later stages of cell wall biosynthesis have been affected, possibly due to the loss of several putative lipoproteins linked to cell wall homeostasis (Table 1 and Table S3). Six of these proteins contain the YkuD domain (PFam PF03734) and are homologous to L-D-transferase (Ldt) transpeptidase enzymes involved in 3-3 cross-linking of the cell wall and in the covalent attachment of proteins to the peptidoglycan (Magnet *et al.*, 2007a,b) (Table S3). Ldt transpeptidases offer an alternative to the standard 4-3 cross-linking catalysed by PBPs (Magnet *et al.*, 2007b). In mycobacteria 3-3 cross-linking is prevalent in stationary phase and there is evidence that these cross-links occur in *Streptomyces* cell

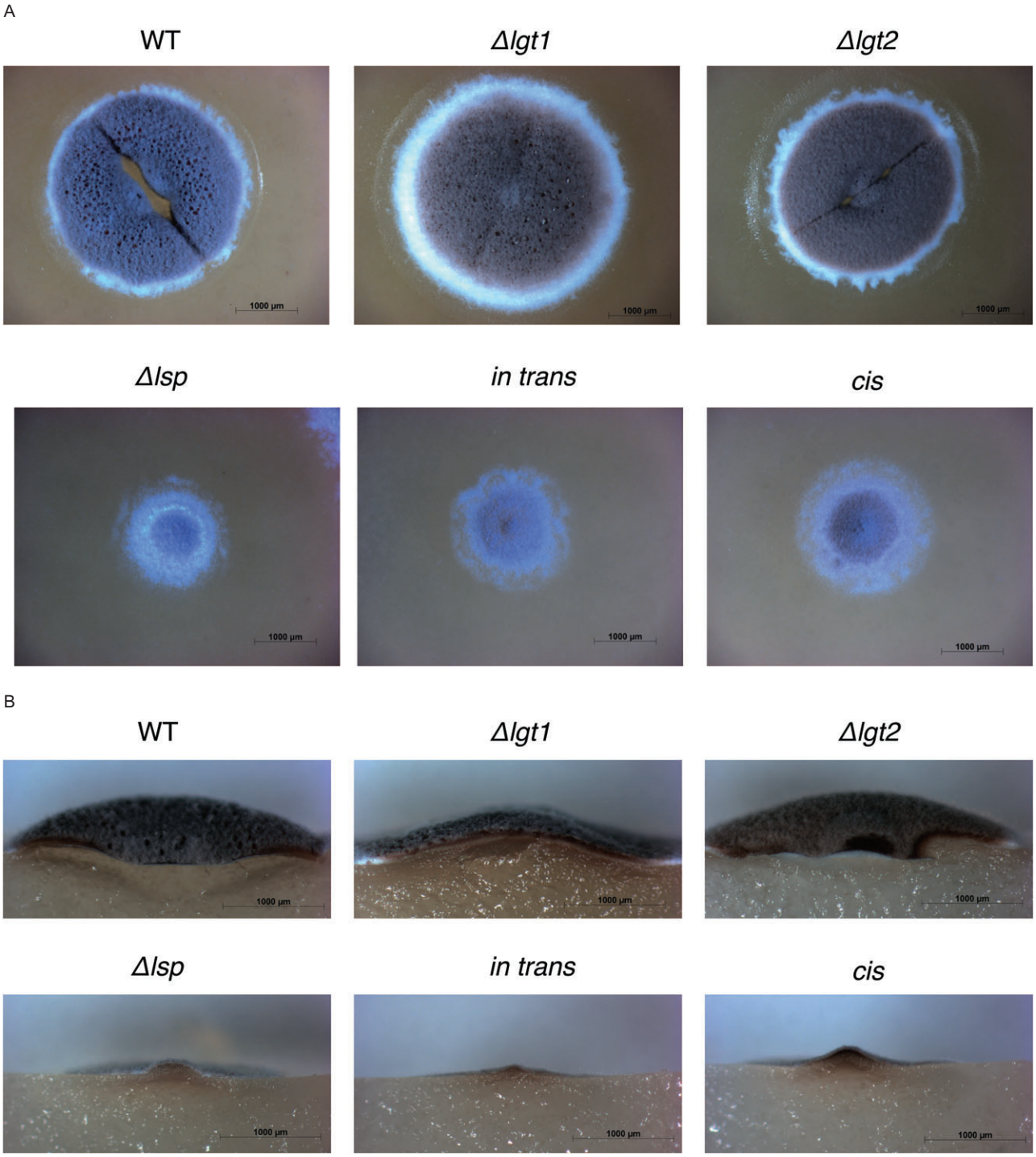

Fig. 1. A. Light microscope images (×40 magnification) of single colonies of *S. coelicolor* wild-type M145, *Δlgt1*, *Δlgt2*, *Δlsp* and *Δlsp* complemented *in trans* and *cis*, as indicated, after 5 days growth on soya flour plus mannitol agar. The *Δlsp* mutant forms small flat colonies compared with the characteristic raised colonies of wild-type M145. The *Δlgt1* and *Δlgt2* mutants are indistinguishable from the wild-type. Complementation of *Δlsp* fails to restore wild-type growth and development.
B. Light microscope images (×40 magnification) of cross-sections of single colonies of *S. coelicolor* wild-type M145, *Δlgt1*, *Δlgt2*, *Δlsp* and *Δlsp* complemented *in trans* and *cis*, as indicated, after 5 days growth on soya flour plus mannitol agar. The *Δlsp* mutant forms small flat colonies compared with the characteristic raised colonies of wild-type M145. Once again the *Δlgt1* and *Δlgt2* mutants are indistinguishable from the wild-type and complementation of *Δlsp* fails to restore wild-type growth and development.

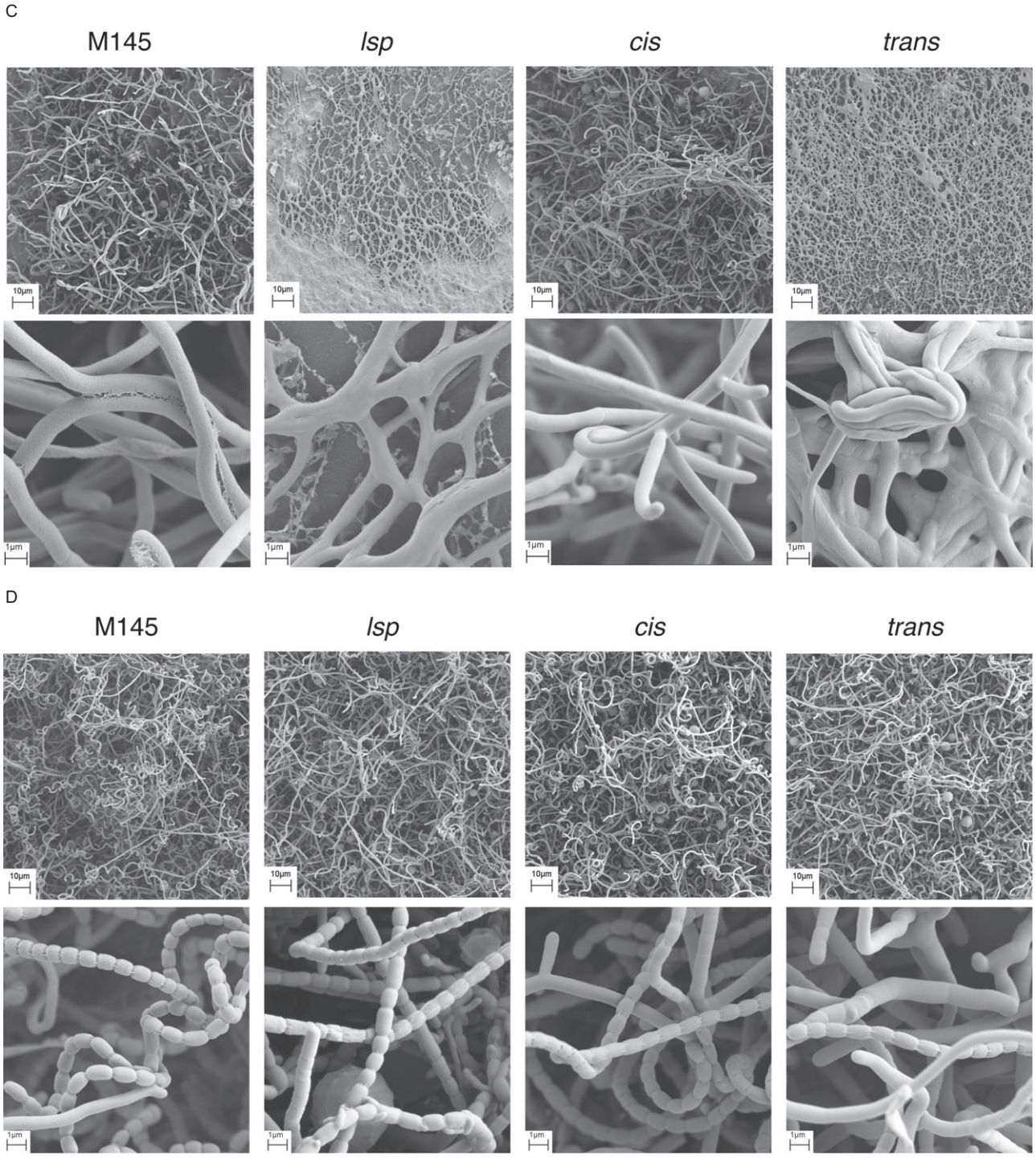

Fig. 1. C. Scanning electron microscopy of *S. coelicolor* wild-type M145, *Δlsp* and *Δlsp* complemented *in trans* and *cis* after 2 days growth on soya flour plus mannitol agar shows that *Δlsp* is delayed in aerial hyphae formation. Complementation *in trans* failed to restore the wild-type phenotype, with only substrate mycelium visible, as observed for the *Δlsp* strain, while *cis* complementation restores aerial hyphae formation to *Δlsp*. The top panels are ×500 magnification and the bottom panels ×5000 magnification.
D. Scanning electron microscopy of *S. coelicolor* wild-type M145, *Δlsp* and *Δlsp* complemented *in trans* and *cis* after 5 days growth on soya flour plus mannitol agar reveals that the *Δlsp* strain is sporulating and looks similar to wild-type at ×500 and ×5000 magnification, although there are noticeably more abnormal sized, and germinating spore compartments in the *Δlsp* spore chains compared with wild-type.

walls (Leyh-Bouille *et al*., 1970; Gupta *et al*., 2010). Recycling of mature peptidoglycan likely results in precursors with stem tetrapeptides that can only be cross-linked 3-3 and the putative lipoprotein Ldt homologues in *S. coelicolor* are most likely involved in the recycling of mature peptidoglycan. The *lgt1* and *lgt2* genes were replaced with apramycin and hygromycin resistance cassettes, respectively, and the resulting strains had no obvious phenotype (Fig. 1). Despite repeated attempts an *lgt* double mutant could not be isolated which may suggest that the function of Lgt is essential in *S. coelicolor*.

Complementation of Δlsp

Complementation *in trans* with wild-type *lsp* under the control of its own promoter only partially restored wild-type growth and development (Fig. 1), suggesting that either complementation *in trans* cannot fully restore Lsp activity or that deletion of *lsp* results in secondary mutations. To investigate this further the wild-type 4A10 cosmid (Redenbach *et al*., 1996), carrying wild-type *lsp* and surrounding genes, was reintroduced into the *Δlsp::apr* strain to restore the wild-type strain. Again, the complemented strain had much smaller colonies than the wild-type (Fig. 1), suggesting that deletion of *lsp* generates one or more additional mutations elsewhere in the genome of *S. coelicolor*, most likely to suppress the lethal effects of deleting *lsp*. The promoters of the vancomycin resistance (*van*) genes have been used previously to show that *chpE* and *femX* are essential genes in *S. coelicolor* (Hong *et al*., 2005; Di Berardo *et al*., 2008). Unfortunately, attempts to deplete *lsp* using the vancomycin inducible *vanJ* promoter were unsuccessful, most likely because this promoter is leaky (M.I. Hutchings, unpublished) and low levels of Lsp are enough to rescue an *lsp* mutant whereas much higher expression of *chpE* and *femX* is required to prevent lethality. Future work will be aimed at attempting to map the secondary mutations in the *lsp* mutant.

Analysing the lipoproteome of S. coelicolor

The pleiotropic phenotype of the *Δlsp* strain could be explained by the loss of some or all of its lipoproteins from the membrane including one or more with essential functions. To identify lipoproteins and further investigate the effects of deleting *lgt1*, *lgt2* and *lsp* on lipoprotein biogenesis, whole-cell lysates were prepared from wild-type, *Δlsp*, *Δlgt1* and *Δlgt2* strains grown on solid growth medium. As a significant proportion of lipoproteins may be Tat substrates, lysates were also prepared from a *ΔtatC* strain to identify Tat-dependent lipoproteins. The lysates were phase partitioned with Triton X-114, to separate hydrophilic proteins into the aqueous phase and lipophilic proteins into the detergent phase, a well-established technique for isolating lipoproteins (Tawaratsumida *et al*., 2009). Proteins from the detergent phase were precipitated with methanol and chloroform and resolved using 2D gel electrophoresis. Comparison of 2D gels loaded with wild-type and *Δlsp* extracts show obvious differences, with many proteins either missing or greatly reduced in the *Δlsp* strain (Fig. 2). The lipoprotein spots identified on 2D gels loaded with wild-type extracts but missing or reduced in 2D gels loaded with extracts from the *Δlsp* strain are shown in Fig. S3. Crucially, these proteins are restored by *in trans* complementation, strongly suggesting they are lipoproteins. This also demonstrates that complementation corrects the defect in lipoprotein biogenesis (Fig. 2), even though it does not restore wild-type growth and development (Fig. 1). In contrast, no differences were observed between the wild-type, *Δlgt1* and *Δlgt2* strains, and no lipoproteins were affected by deletion of *lgt1* or *lgt2* (results not shown). The most likely explanation is thus that the Lgt enzymes can effectively complement one another. There is no evidence to suggest that the Lgt enzymes are pathway specific for Tat- or Sec-dependent lipoproteins as neither subset are selectively affected in either the *lgt1* or the *lgt2* deletion strains. It is intriguing that *S. coelicolor* encodes two Lgt orthologues (with 62% amino acid identity) since there appears to be only a single Lgt encoded in all other *Streptomyces* species for which genome sequence data are available, with the exception of *Streptomyces clavuligerus*, which has a second Lgt encoded on its megaplasmid. The fact that *S. coelicolor* Lgt2 is encoded by a gene in an arm of the *S. coelicolor* chromosome may suggest that this gene has been acquired by horizontal gene transfer and has been retained because of the apparently essential nature of Lgt function in *S. coelicolor.*

MALDI TOF analysis of the proteins that were either missing or reduced in the *Δlsp* samples revealed that 29 of the putative lipoproteins identified in the bioinformatic analysis were present in the lipophilic fraction of the wild-type, suggesting they are indeed lipoproteins (Table 2). Six of these lipoproteins were missing from the *ΔtatC* strain (Table 2 and Fig. S1), three of which (SCO1639, SCO2780 and SCO7677) are known Tat substrates (Widdick *et al*., 2006), while an additional one (SCO2828) was confirmed here using agarase as a reporter enzyme (Widdick *et al*., 2008). To further investigate processing of Tat-dependent lipoproteins the coding sequences of SCO1639 and SCO2780, the two most highly expressed Tat-lipoproteins identified in this study, were PCR-amplified along with their promoters and with six histidine codons at their 3′ ends. The genes were introduced into M145, *ΔtatC* and *Δlsp* in single copy. Membrane and cytoplasmic fractions, along with total proteins isolated from culture supernatants, were then probed by immunoblotting with anti-His antibodies. Both lipoproteins were present in the membranes of M145

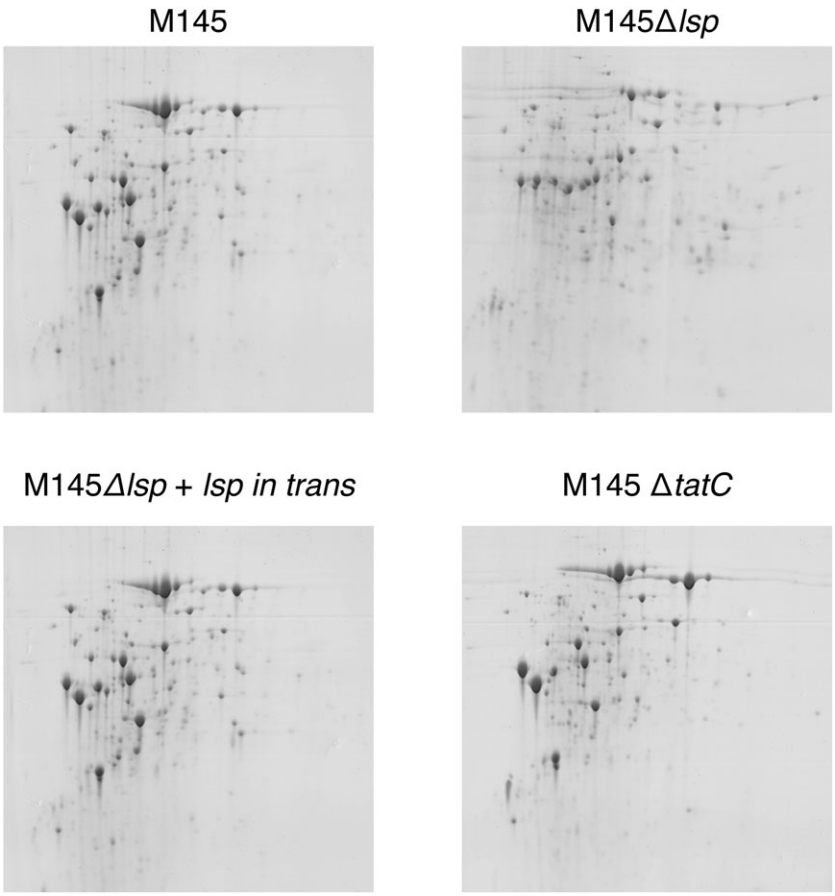

Fig. 2. Two-dimensional gel analysis of Triton X-114 extracted membrane proteins from *S. coelicolor* wild-type M145, *Δlsp*, *Δlsp* complemented *in trans* and *ΔtatC* after 5 days growth on TSB/YEME agar. These images show clear differences in the protein profiles of the wild-type and *Δlsp* strains, with most of the missing or reduced spots identified as putative lipoproteins (Table 2). Significantly, these proteins are restored by *in trans* complementation with *lsp* demonstrating that the defect in lipoprotein biogenesis is corrected but wild-type growth and development are not restored.

and the cytoplasm of the *ΔtatC* strain, suggesting they are Tat-dependent (as shown previously for SCO2780) and some SCO1639 was also present in the culture supernatant of the wild-type strain (Fig. 3). Membrane localization of the putative Tat dependent lipoprotein SCO3484 was also perturbed in the *ΔtatC* strain, suggesting it too is Tat-dependent. SCO1639 was also present in membranes and the culture supernatant of the *Δlsp* strain and its shifted size suggests the presence of an uncleaved signal peptide (Fig. 3), confirming its lipoprotein nature. Both SCO2780 and SCO3484 were notably absent from all *Δlsp* fractions. These results provide further evidence that SCO1639, SCO2780 and SCO3484 are Tat-dependent lipoproteins and also suggests that at least one unprocessed Tat-dependent lipoprotein is present in the membrane of the *Δlsp* strain, whereas other lipoprotein precursors may be removed by proteolysis. Different effects on the processing of individual lipoprotein precursors have been observed in other studies with mutants in the lipoprotein biosynthesis pathway (Hutchings *et al.*, 2009). The control, Sec-dependent lipoprotein CseA (Hutchings *et al.*, 2006b) is present, as expected, in membranes from both M145 and *ΔtatC* but is absent from all *Δlsp* fractions (Fig. 3).

SCO1639 is a putative peptidyl-prolyl *cis trans* isomerase (PPIase), likely involved in the folding of secreted proteins and is orthologous to the previously characterized *Streptomyces chrysomallus* protein FKBP-33 (Pahl and Keller, 1994). SCO1639 therefore likely has a comparable function to the essential lipoprotein foldase PrsA in *B. subtilis* which is also present and functional in the membrane of a *Δlsp* strain of *B. subtilis* (Tjalsma *et al.*, 1999). However, SCO1639 and PrsA belong to different PPIase subfamilies and *SCO1639* can be deleted in *S. coelicolor* without any adverse effects on growth or morphology (D.A. Widdick, unpublished), consistent with the non-essentiality of *tatC*. Intriguingly, *SCO1639* is co-transcribed with a second PPIase gene, *SCO1638* that lacks a signal sequence but could possibly 'hitch-hike' out through Tat by complexing with SCO1639. Unfortunately, all attempts to detect SCO1638 either in the cytoplasmic and membrane fractions or culture supernatants of *S. coelicolor* were unsuccessful, suggesting it is not produced under the conditions used in this study.

Combined with the bioinformatic analysis, the data presented here suggest that Tat is involved in translocating significant numbers of lipoproteins in *S. coelicolor* and provides the first confirmation that experimentally verified lipoproteins are Tat-dependent in bacteria. Tat is therefore a major pathway for both protein secretion (Widdick *et al.*, 2006) and lipoprotein translocation in *Streptomyces*, with

Table 2. Lipoproteins identified by 2D analysis.

Name	Function	Signal peptide prediction	Detected in Δ*tatC*	Agarase[a]
SCO0472	SBP, Interpro 11044	Sec	Y	Not tested
SCO0474	SBP, Interpro 11044	Sec	Y	–
SCO0494	SBP, iron-siderophores	Sec	Y	–
SCO1655	SBP, PF00496 family 5	Sec	Y	Not tested
SCO2008	SBP, branched chain amino acids	Sec	Y	Not tested
SCO2231	SBP, maltose	Sec	Y	Not tested
SCO2795	SBP, PF01547	Sec	Y	Not tested
SCO2978	SBP, PF01547	Sec	Y	Not tested
SCO3966	Trx-like fold, cytochrome *c* biogenesis	Sec	Y	Not tested
SCO4884	SBP PF02608, Bmp/ribonucleosides	Sec	Y	Not tested
SCO4885	SBP PF02608, Bmp/ribonucleosides	Sec	Y	–
SCO5113, BldKB	SBP, oligopeptides	Sec	Y	–
SCO5260	SBP, PF00497	Sec	Y	Not tested
SCO5430	SBP, PF01547	Sec	Y	Not tested
SCO5477	SBP, PF00496	Sec	Y	Not tested
SCO5776	SBP, PF00497	Sec	Y	Not tested
SCO6009	SBP	Sec	Y	–
SCO6065	SBP, glycine betaine-related	Sec	Y	Not tested
SCO6451	SBP, PF00496	Sec	Y	Not tested
SCO6644	SBP, PF00496	Sec	Y	–
SCO6979	SBP	Sec	Y	Not tested
SCO7028	SBP, PF01547	Sec	Y	Not tested
SCO7399	SBP, iron siderophore	Sec	Y	–
SCO1639	Peptidyl-prolyl cis-trans isomérase	Tat	N	+
SCO2404	SBP	Tat	N	Not tested
SCO2780, DesE	SBP, iron siderophore	Tat	N	+
SCO2828	SBP, PF00497	Tat	N	+
SCO4934	Putative L-D-transpeptidase	Tat	N	Not tested
SCO7677	SBP, PF00496	Tat	N	+

a. Assay for agarase activity as described previously (Widdick *et al.*, 2008).

similar numbers of lipoproteins also predicted to be Tat substrates in *Streptomyces griseus*, *Streptomyces avermiltis* and *Streptomyces scabies* (Table S4). This is analagous to the situation in the archaeon *Haloferax volcanii* (Giménez *et al.*, 2007), in which Tat is the major translocase for lipoproteins. However, the lipoprotein biogenesis pathway has not been identified in archaea and is not homologous to that of bacteria. Many actinomycetes are

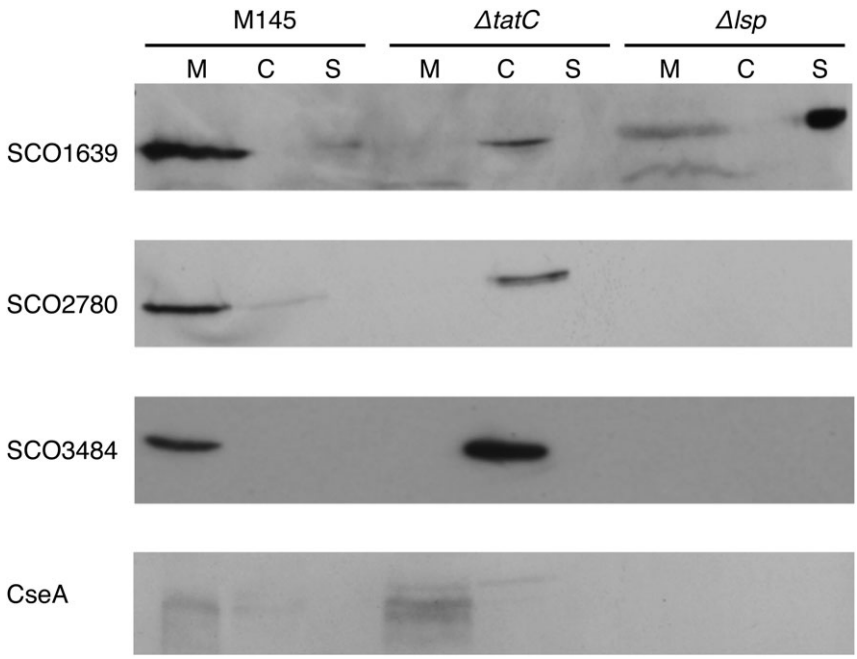

Fig. 3. To further investigate putative Tat-dependent lipoproteins, SCO1639 (FkpA), SCO2780 (DesE) and SCO3484 were expressed with hexa-his tags in *S. coelicolor* wild-type M145 and in the *Δlsp* and *ΔtatC* strains. Proteins were extracted from the cytoplasmic (C) and membrane (M) fractions and the growth medium (culture supernatants, S) of these strains and probed by immunoblotting with monoclonal anti-his antibodies. All three lipoproteins are present in the membranes of the wild-type strain and the cytoplasm of the *tatC* strain suggesting they are Tat-dependent. Only SCO1639 is present in the membrane of the *lsp* strain and is larger in size, consistent with the presence of its signal sequence. The bottom panel shows immunoblotting experiments against the experimentally verified Sec-dependent *S. coelicolor* lipoprotein CseA, detected with anti-CseA antibodies.

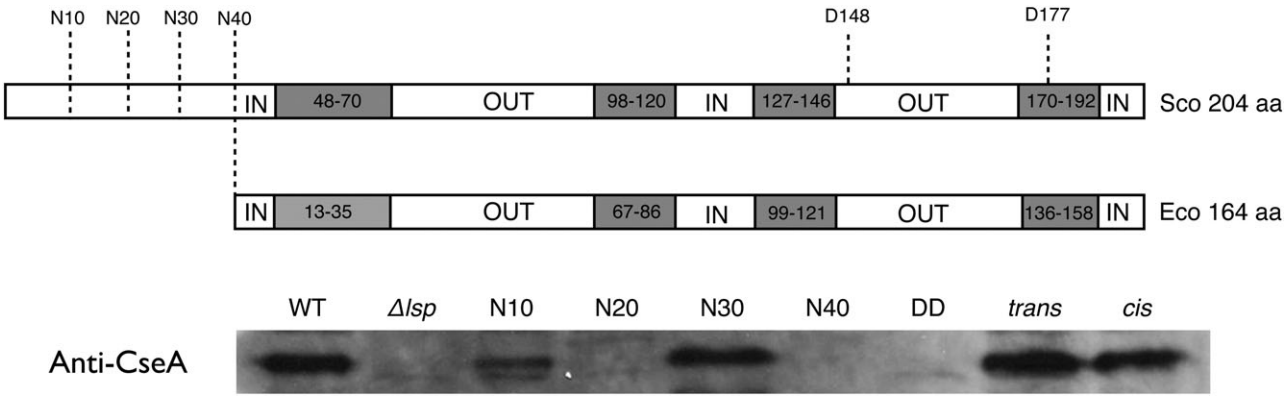

Fig. 4. The top panel shows a diagrammatic representation of *S. coelicolor* and *E. coli* Lsp enzymes. The positions at which the *S. coelicolor* truncated N10, N20, N30 and N40 Lsp enzymes start are marked, with each truncated protein starting with methionine. The predicted or known transmembrane helices are highlighted, as are the conserved aspartate residues required for catalytic activity (D148 and D177 in *S. coelicolor*). The bottom panel shows immunoblotting experiments against the experimentally verified lipoprotein CseA. Lanes contain membrane fractions from wild-type strain M145, *Δlsp* and *Δlsp* expressing truncated N40 Lsp, N30 Lsp, N20 Lsp, N10 LspP, DD (D148A/D177A) Lsp, in which both conserved aspartate residues are changed to alanine, full-length (FL) Lsp expressed *cis* or *in trans*. Cytoplasmic and secreted proteins were also isolated from each strain but CseA was not detected in any of these samples (data not shown).

predicted to translocate more than 10% of their putative lipoproteins via Tat (Hutchings *et al*., 2009; Shruthi *et al*., 2010). Significantly, the widespread use of Tat in *Streptomyces* potentially provides a mechanism for overproduction and secretion of fully folded heterologous proteins and could also be harnessed for cell surface display, using Tat lipoprotein signal sequences to direct proteins to the extracytoplasmic face of the cell membrane. With *c*. 50 Tat lipoprotein signal sequences in *S. coelicolor* this could solve several problems associated with current bacterial cell surface display systems, notably the inability to display more than one protein and the inability to display very large proteins or protein complexes (Wu *et al*., 2008). It should be noted that any lipoproteins with essential functions must be Sec-dependent because Tat is non-essential in *S. coelicolor*.

The N-terminal extension is required for Lsp activity in S. coelicolor

Alignment of the primary Lsp sequences from diverse bacteria revealed that the Lsp enzymes of some actinomycetes contain an N-terminal extension not present in other bacteria (Fig. S2). This extension is upstream of the first putative transmembrane helix and is predicted to be cytoplasmic, suggesting it could interact with a C-terminal cytoplasmic extension found in the Lgt enzymes of actinomycetes (Fig. S1) (Rezwan *et al.* 2007; Sutcliffe and Hutchings, 2007). To determine whether this N-terminal extension is required for activity in *S. coelicolor* Lsp we made a series of deletions in the coding sequence of *lsp* to express enzymes which start at amino acid 10 (N10), 20 (N20), 30 (N30) and 40 (N40), where the first residue in N40 Lsp aligns with the first amino acid in *E. coli* Lsp. We also altered the two putative active site aspartate residues (D148 and D177) to alanine to make an inactive form of the Lsp protein (Figs 4 and S2). Constructs carrying the altered *lsp* alleles were introduced into the unmarked *Δlsp* mutant in single copy and under the control of the native *lsp* promoter to make strains identical to the *in trans* complemented strain except for the deleted codons. Complementation was tested by immunoblotting membrane fractions with antibodies against CseA (Hutchings *et al*., 2006b), and Lsp enzymes were judged to be active if the mature form of CseA was present in the membrane. The processed form of CseA is clearly present in the membranes of the strain expressing N10 Lsp and absent from strains expressing the D148A/D177A mutant Lsp and the N20, N30 and N40 truncated Lsp. Intriguingly, a slightly larger CseA protein is present in the strain expressing N30 Lsp, consistent with the presence of its signal peptide (Fig. 4). This suggests that the N20, N30 and N40 Lsp enzymes are either unstable or inactive and that only the full-length and N10 Lsp enzymes are active. Unfortunately, an antibody raised against a synthesized peptide fragment [KLEHHEPIEIIGDWLRFA] located in the extracytoplasmic loop between transmembrane helices 1 and 2 in *S. coelicolor* Lsp (residues 72 to 89) failed to detect Lsp in *S. coelicolor* membrane fractions. Attempts to colocalize the Lsp and Lgt enzymes using the fluorescent reporters eGFP and mCherry were unsuccessful, because the proteins are apparently produced at very low level. However, the results presented here suggest that the N-terminal Lsp extensions common to actinomycetes are required for activity, at least in *S. coelicolor* Lsp.

In summary, this work has shown that Tat plays a major role in the translocation of lipoproteins across the cyto-

plasmic membrane in *S. coelicolor* with almost one quarter of lipoprotein precursors exported through Tat. This has implications for biotechnology because Tat lipoprotein signal sequences could be exploited for heterologous cell surface display in *Streptomyces*. Our data also suggest that disrupting lipoprotein biogenesis has a severe and detrimental effect on the growth of *S. coelicolor* with additional, as yet unidentified, mutations arising in an *lsp* mutant. *S. coelicolor* is unusual among bacteria in having two copies of the *lgt* gene and despite repeated attempts we were unable to delete both copies of *lgt* in the same strain, which supports the hypothesis that disrupting lipoprotein biogenesis is highly deleterious to the fitness of *S. coelicolor* and may even be lethal to this bacterium. In future work it will be important to identify the secondary mutations in the *S. coelicolor lsp* mutant, which likely play a role in suppressing the effects of deleting *lsp*. This may give insights into why disruption of *lsp* has such a drastic effect on *S. coelicolor* while apparently having very little effect on the growth and morphology of other Gram-positive bacteria.

Experimental procedures

Bioinformatic identification of lipoproteins

All proteins in the *S. coelicolor* database (http://strepdb.streptomyces.org.uk) with a cysteine residue in the first 50 amino acids were matched against the G + LPP pattern for the recognition of typical lipoprotein signal sequences (Sutcliffe and Harrington, 2002; Rahman *et al*., 2008) and a revision (<[MV]-X(0,37)-[RK]-{DERK}(6,20)-[LIVMFESTAGP]-[LVIAMFTG]-[PIVMSTAFGC]-[AGS]-C) (Sutcliffe and Harrington, 2002; Hutchings *et al*., 2009), which allows for the recognition of potential lipoproteins with long signal peptide N-regions. Additional putative lipoproteins were recovered by searching with the Prosite prokaryotic membrane lipoprotein profile (PS51257). All of the proteins retained in the above filter were submitted to SignalP version 3.0 (Bendtsen *et al*., 2004), Phobius (Käll *et al*., 2004), LipoP (Juncker *et al*., 2003) and PredLipo (Bagos *et al*., 2008). Integrating data from SignalP, Phobius and LipoP is highly accurate for the recognition of Gram-positive bacterial lipoproteins (Rahman *et al*., 2008). Pred-Lipo is also a highly sensitive and specific tool for the identification of Gram-positive bacterial lipoproteins (Bagos *et al*., 2008; our unpublished observation). TatFind (Rose *et al*., 2002) and TatP (Bendtsen *et al*., 2005) were used to identify which putative lipoproteins are also likely to be Tat substrates. The primary sequences of proteins passing this analysis were individually inspected to exclude false positive Tat substrates.

Strains, plasmids, primers and growth conditions

The strains, plasmids and primers used in this study are listed in Table 3. *E. coli* strains were routinely grown in Lennox broth (LB) or LB without NaCl to select for hygromycin resistance, and supplemented with arabinose as necessary. *S. coelicolor* strains were grown on soya-flour mannitol (SFM) agar, Difco nutrient broth agar (BD Diagnostics) and a 50:50 mix of tryptone soya broth (TSB – Oxoid) and yeast extract-malt extract (YEME) agar. Liquid cultures were grown in Difco nutrient broth or a 50:50 mix of TSB and YEME. All growth media recipes were taken from Kieser *et al*. (2000). The agarase reporter assays were performed as described previously (Widdick *et al*., 2006) using signal peptides from the Tat substrates listed in Table 2. The *lsp* complementation constructs were synthesized by Genscript and subcloned into pSET152 (Table 3). The expression vectors for His-tagged Tat-dependent lipoproteins were constructed by PCR amplifying the *SCO1639*, *SCO2780* and *SCO3484* genes with their respective forward and reverse primers, where the latter incorporated six histidine codons before the stop codon.

Gene deletions

Streptomyces coelicolor mutants were constructed using a PCR-targeting method (Gust *et al*., 2003), as described previously (Hutchings *et al*., 2006a). The *lsp* gene was replaced on cosmid 4A10 by electroporating the cells with an *apr–oriT* cassette, PCR-amplified using primers lsp KOfor and lsp KOrev (Table 3). The *lgt1* gene was replaced on cosmid 4G6 by electroporating the cells with an *apr–oriT* cassette, PCR-amplified using primers lgt1 KOfor and lgt2 KOrev. The *lgt2* gene was replaced on cosmid 8E7 by electroporating the cells with an *hyg-oriT* cassette, PCR-amplified using primers lgt1 KOfor and lgt2 KOrev. The knockout cosmids were checked relative to wild-type by restriction digestion with SacI and BamHI, and compared with a predicted pattern generated by the electronic restrict tool at http://*streptomyces*.org.uk). All cosmids were also checked by PCR with the relevant test primers (Table 3). The 4A10*lsp::apr* and *4G6lgt1::apr* cosmids were used to transform *E. coli* strain ET12567 containing the driver plasmid pUZ8002 and the resulting strains were conjugated with *S. coelicolor* M145. The 8E7*lgt2::hyg* cosmid failed to give rise to clean *lgt2* deletion mutants, presumably due to transposable elements on cosmid 8E7. As a result, a 6087 bp NotI restriction fragment, containing *lgt2::hyg* plus flanking regions, was excised and subcloned into the NotI cut vector pBSIIKS. The *bla* gene in the pBSIIKS backbone was replaced with the *neo* gene by PCR-targeting to make a kanamycin resistance plasmid and then conjugated via ET12567/pUZ8002 into M145. Exconjugants were selected by identifying colonies that were apramycin (*lsp* and *lgt1*) or hygromycin resistant (*lgt2*) ($Apra^R$ or Hyg^R) and kanamycin sensitive (Kan^S). Exconjugants were confirmed by PCR analysis using all combinations of the respective test primers with primers P1 and P2 (Table 3). An unmarked, in-frame deletion of *lsp* was constructed by transforming *E. coli* strain BT340 with the cosmid 4A10*lsp::apr* at 30°C and inducing FLP recombinase in this strain at 42°C to remove *apr–oriT* (Gust *et al*., 2003). This cosmid was then PCR-targeted to replace the ampicillin resistance gene *bla* with a hygromycin resistance cassette (containing *oriT*) that was amplified with primers blaFOR and blaREV (Table 3). Hygromycin resistant, ampicillin sensitive colonies were selected and confirmed by PCR with bla TESTfor and bla TESTrev (Table 3). The altered cosmid was then used to transform

Table 3. Strains, plasmids and primers used in this study.

Strain		References
S. coelicolor		
M145	SCP1⁻, SCP2⁻ *S. coelicolor* wild-type strain	Kieser *et al.* (2000)
BJT1000	M145*Δlsp::apr*	This work
BJT1001	M145*Δlsp*	This work
BJT1002	M145*Δlgt1::apr*	This work
BJT1003	M145*Δlgt2::hyg*	This work
BJT1004	M145Δlsp + *lsp cis*	This work
BJT1005	M145Δlsp + *lsp in trans*	This work
BJT1006	M145Δlsp + N10 *lsp*	This work
BJT1007	M145Δlsp + N20 *lsp*	This work
BJT1008	M145Δlsp + N30 *lsp*	This work
BJT1009	M145Δlsp + N40 *lsp*	This work
TP4	M145*ΔtatC*	Widdick *et al.* (2006)
DW1000	M145 + SCO1639-His	This work
DW1001	BJT1001 + SCO1639-His	This work
DW1002	TP4 + SCO1639-His	This work
DW1003	M145 + SCO2780-His	This work
DW1004	BJT1001 + SCO2780-His	This work
DW1005	TP4 + SCO2780-His	This work
DW1006	M145 + SCO3484-His	This work
DW1007	BJT1001 + SCO3484-His	This work
DW1008	TP4 + SCO3484-His	This work
E. coli		
BW25113 (pIJ790)	BW25113 containing λ RED recombination	Gust *et al.* (2003)
ET12567 (pUZ8002)	*dam dcm* strain containing helper plasmid pUZ8002	Gust *et al.* (2003)
BT340	DH5α + plasmid pIJ790	Gust *et al.* (2003)
Plasmid		
pSET152	Integrative *Streptomyces* vector	Kieser *et al.* (2000)
pMS82	Integrative *Streptomyces* vector	Kieser *et al.* (2000)
pBT100	pSET152 + full-length *lsp*	This work
pBT101	pSET152 + D148A, D177A *lsp*	This work
pBT102	pSET152 + N10 *lsp*	This work
pBT103	pSET152 + N20 *lsp*	This work
pBT104	pSET152 + N30 *lsp*	This work
pBT105	pSET152 + N40 *lsp*	This work
pTDW186	pSET152 + SCO1639-6xHis	This work
pTDW187	pSET152 + SCO2780-6xHis	This work
pTDW188	pSET152 + SCO3484-6xHis	This work
Primer		
lsp KOfor	tcgtgctcagtcaaggacctaggctgagggactcacgtgattccggggatccgtcgacc	This work
lsp KOrev	gacaaccagtccctgtggacagccggaccggaggggtcatgtaggctggagctgcttc	This work
lsp TESTfor	tcgtgctcagtcaaggacct	This work
lsp TESTrev	gacaaccagtccctgtggac	This work
lgt1 KOfor	gcgccccccgtccgacacggtagcgtcgaccctgccatgattccggggatccgtcgacc	This work
lgt1 KOrev	taccgggcgccctcggcggtgtgtcttgcgggcgggtcatgtaggctggagctgcttc	This work
lgt1 TESTfor	gcgccccccgtccgacacgg	This work
lgt1 TESTrev	taccgggcgccctcggcggt	This work
lgt2 KOfor	gaaaccctccacgacctcgaccaaggctctcgatcatgattccggggatccgtcgacc	This work
lgt2 KOrev	ttcgcaccagcaccgccggtcgcctgtacgagcgcatcatgtaggctggagctgcttc	This work
lgt2 TESTfor	gaaaccctccacgacctcg	This work
lgt2 KOrev	ttcgcaccagcaccgccggt	This work
P1	attccggggatccgtcgacc	This work
P2	tgtaggctggagctgcttc	This work
blaFOR	ccctgataaatgcttcaataatattgaaaaaggaag	This work
blaREV	aatcaatctaaagtatatatgagtaaacttggtctgacag	This work
bla TESTfor	cctagatccttttaaattaaaaatg	This work
bla TESTrev	tcaaatatgtatccgctcatgagac	This work
SCO1639 FORWARD	ggcgcgggatccccgggtctgagccgggcggcat	This work
SCO1639 REVERSE	cgcgccggatcctcagtgatggtgatggtggtgcatcttcgcgaggatgtccacg	This work
SCO2780 FORWARD	ggcgcgggatcccctggacaagatctgaggttag	This work
SCO2780 REVERSE	cgcgccggatcctcagtgatggtgatggtggtggccgaccttcttggcgttctcg	This work
SCO3484 FORWARD	ggcgcgggatccgacctcgaggcggtcgtgagctg	This work
SCO3484 REVERSE	cgcgccggatcctcagtgatggtgatggtggtgcatgaccttctcgatgccgtcc	This work

E. coli ET12567/pUZ8002, and conjugated into M145*lsp::apr*. Selection for single exconjugants involved picking colonies that were Hyg^R, Kan^R and Apra^s. After growth on SFM agar in the absence of antibiotics, double exconjugants were selected by identifying colonies that were Hyg^S, Kan^s and Apra^S. Gene deletion was confirmed by PCR, as above, with a characteristic 81 bp sequence showing that the *apr–oriT* cassette had been excised (Gust *et al*., 2003).

Complementation

Full-length wild-type and mutated (D148A, D177A) *lsp* and the N-terminal *lsp* truncations were synthesized by Genscript such that the first codon was in the same position as the annotated *lsp* start codon with an additional 300 bp upstream DNA, containing the *lsp* promoter. Each allele was subcloned into pSET152 to make plasmids pBT100-105 and each plasmid was used to transform ET12567/pUZ8002 and then conjugated into M145*lsp::apr*. Exconjugants were selected using apramycin resistance. Reintroducing the wild-type copy of *lsp* back into strain M145*lsp::apr* involved replacing the *bla* gene in the backbone of cosmid 4A10 with the hygromycin resistance cassette, as described above. The altered 4A10 cosmid was then used to transform *E. coli* ET12567/pUZ8002, and conjugated into M145*lsp::apr*. Double exconjugants were selected by identifying colonies that were Hyg^S and Apra^S and tested by PCR, using the lsp TESTfor and Lsp TESTrev primers, as described above.

Immunoblotting

For subcellular fractionation, *S. coelicolor* cultures were grown for 15 h at 30°C in TSB/YEME. Crude cell extracts were obtained by harvesting the mycelium, washing in 1 ml of buffer A (100 mM Tris pH 8.0, 50 mM NaCl), and sonicating for 5 s, on ice, three times, in 200 µl buffer A plus EDTA-free protease inhibitor (Roche). Cell debris was removed by centrifuging at 4000 r.p.m. for 5 min at 4°C followed by ultracentrifugation at 80 000 r.p.m. at 4°C, and the supernatant containing cytoplasmic proteins was removed and frozen at –20°C. The remaining pellet was washed in 200 µl buffer A and centrifuged for 1 h at 80 000 r.p.m. at 4°C. The supernatant was discarded, and the membrane containing pellet was resuspended in buffer A plus 1% Sarcosyl and stored at –20°C. Protein fractions were separated on a 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis gel run at 200 V for 1.5 h and transferred to polyvinylidene difluoride (PDVF) membrane (pre-soaked in methanol) using a Bio-Rad semi-dry transfer cell, set up according to the manufacturers instructions and run at 15 V for 1 h. The membrane was incubated for 15 h (overnight) in blocking solution (Tris buffered saline + 1% Tween 20 + 5% skimmed milk powder). Immunoblotting was carried out as described previously (Hutchings *et al*., 2006b) with 1/1250 dilutions of anti-CseA antibody and 1/5000 dilutions of horseradish peroxidase-linked goat anti-rabbit IgG antibody (Promega). Membranes were developed using the ECL system (GE Healthcare), exposed to X-ray film for between 30 s and 5 min and developed using an Xograph X-ray film processor.

Microscopy

Brightfield images were acquired using a Zeiss M2 Bio Quad SV11 stereomicroscope. The samples were illuminated with a halogen lamp and reflected-light images captured with an AxioCam HRc CCD camera and AxioVision software (Carl Zeiss, Welwyn Garden City, UK). For scanning electron microscopy samples were mounted on an aluminium stub using Tissue Tek^R (BDH Laboratory Supplies, Poole, England). The stub was then immediately plunged into liquid nitrogen slush at approximately –210°C to cryo-preserve the material. The sample was transferred onto the cryostage of an ALTO 2500 cryo-transfer system (Gatan, Oxford, England) attached to a Zeiss Supra 55 VP FEG scanning electron microscope (Zeiss SMT, Germany). Sublimation of surface frost was performed at –95°C for three minutes before sputter coating the sample with platinum for 3 min at 10 mA, at colder than –110°C. After sputter-coating, the sample was moved onto the cryo-stage in the main chamber of the microscope, held at approximately –130°C. The sample was imaged at 3 kV and digital TIFF files were stored.

Sample preparation for 2D gels

TSB/YEME agar plates covered with sterile cellophane discs were inoculated with ~10^5 spores of M145, TP4, M145*lsp* or M145*lsp* + pBT100. After 48 h the plates were harvested and the biomass frozen at –80°C. The biomass was subsequently thawed and resuspended in sterile Oxoid phosphate buffered saline (PBS; Dulbecco A) plus Complete EDTA-free protease inhibitor (Roche). The cells were lysed by sonication then 5 ml of the sonicate was mixed with 5 ml of Triton X-114 and 40 ml of sterile PBS followed by incubation on ice for 2 h with mixing after one hour. Cellular debris was pelleted by centrifugation at 3345 *g* at 4°C for 30 min. The supernatant was carefully decanted and distributed among three 15 ml falcon tubes and incubated at 37°C for 30 min to allow phase separation of the Triton X-114 and the aqueous phase. The phases were separated by centrifugation at 3345 *g* for 30 min at room temperature. To ensure as little contamination with hydrophilic proteins as possible the upper phase was removed and replaced by an equal volume of fresh PBS then phases mixed and the tubes incubated on ice for 30 min to allow the two phases to integrate. This was followed by a 30 min incubation at 37°C to allow phase separation again, followed by centrifugation as described above. The upper phase was discarded and to the lower phase 4 volumes of methanol was added with mixing, followed by 1 volume of Chloroform with mixing, followed by 3 volumes of analytical grade H_2O with mixing. The tubes were centrifuged at 3345 *g* at room temperature for 30 min to allow phase separation and the upper layer was subsequently carefully discarded. The proteins were then pelleted by the addition of 4 volumes of methanol with mixing and centrifugation at 3345 *g* for 30 min at room temperature. Pellets from the same strain were washed with ethanol, pooled, and resuspended in IEF sample buffer (8 M urea, 2 M thiourea, 0.5% CHAPS, 0.2% DTT). Samples were then treated as described for 2D gel analysis (Widdick *et al*., 2006) with 250 µg protein loaded onto each IEF strip.

Acknowledgements

We thank Paul Thomas for assistance with light and confocal microscopy in the Bioimaging suite at the University of East Anglia (UEA), Kim Findlay for scanning electron microscopy at the John Innes Centre (JIC), Mike Naldrett and Gerhardt Saalbach for MALDI-TOF analysis of 2D gel spots at the JIC (Widdick *et al*., 2006). This work was funded by the Biotechnology and Biological Sciences Research Council (grant numbers F009224/1 and F009429/1 and a PhD studentship to B.J.T.). M.I.H. is a Research Councils UK Academic Fellow.

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