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Aerial development in *Streptomyces coelicolor* requires sortase activity

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Summary

***Streptomyces coelicolor* is a multicellular bacterium whose life cycle encompasses three differentiated states: vegetative hyphae, aerial hyphae and spores. Among the factors required for aerial development are the 'chaplins', a family of eight secreted proteins that coat the surface of aerial hyphae. Three chaplins (the 'long' chaplins, ChpA, B and C) possess an LAXTG-containing C-terminal sorting signal and are predicted sortase substrates. The five remaining 'short' chaplins are presumed to be associated with the cell surface through interactions with the long chaplins. We show here that two sortase enzymes, SrtE1 and SrtE2, cleave LAXTG-containing peptides at two distinct positions *in vitro*, and are required for cell wall anchoring of ChpC *in vivo*. *srtE1/E2* double mutants are delayed in aerial hyphae formation, do not sporulate and fail to display all short chaplins on their aerial surfaces. Surprisingly, these mutant characteristics were not shared by a long chaplin mutant, which exhibited only modest delays in aerial development, leading us to revise the current model of chaplin-mediated aerial development. The sortase mutant phenotype, instead, appears to stem from an inability to transcribe aerial hyphae-specific genes, whose products have diverse functions. This suggests that sortase activity triggers an important, and previously unknown, developmental checkpoint.**

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Introduction

The Gram-positive bacterial cell wall is composed of peptidoglycan layers, interspersed with glycopolymers of teichoic acid and lipoteichoic acid (Silhavy *et al.*, 2010). These macromolecules form a protective barrier and provide structural integrity for the cell. The cell surface is also home to a wide variety of proteins that confer sensory and interactive properties to the cell wall. A subset of these proteins is covalently anchored to the peptidoglycan scaffolding through the activity of sortase enzymes. Sortases are cell membrane-anchored cysteine transpeptidases that cleave specific motifs within the C-terminal 'sorting signal' of their substrates (Marraffini *et al.*, 2006). The sorting signal comprises a sortase-specific pentapeptide motif followed by a stretch of hydrophobic amino acids, which is flanked at the extreme C-terminus by several positively charged residues. Following substrate cleavage, sortases catalyse a transpeptidation reaction that attaches substrate proteins to the peptide cross bridge of nascent peptidoglycan, covalently linking them to the growing cell wall (Marraffini *et al.*, 2006).

Sortase enzymes are found in most Gram-positive bacteria; however, the number of sortases and substrates can vary considerably between different bacteria. For example, *Clostridium botulinum* is predicted to encode a single sortase and substrate (Comfort and Clubb, 2004), while *Listeria monocytogenes 4b* has two sortases and 51 predicted substrates (Bierne *et al.*, 2002; 2004; Boekhorst *et al.*, 2005), many of which are important for virulence. Sortases have been studied most extensively in *Staphylococcus aureus*, which encodes two sortase enzymes, SrtA and SrtB. SrtA is responsible for anchoring proteins whose functions include IgG-binding (protein A) and host tissue adherence (e.g. collagen- and fibronectin-binding proteins) (Mazmanian *et al.*, 1999), while SrtB targets proteins involved in iron sequestration (IsdC) (Mazmanian *et al.*, 2002). In addition to targeting substrates with discrete functions, these two sortases also cleave distinct pentapeptide sequences: SrtA specifically recognizes LPXTG-containing sorting signals, while SrtB targets NPQTN-containing sequences (Marraffini *et al.*, 2006). The role of sortases in bacterial pathogenicity and development has recently expanded to include the assembly of Gram-positive pili in a variety of bacteria (Mandlik *et al.*,

2008a; Hendrickx *et al*., 2011) including *Corynebacterium* (Swaminathan *et al*., 2007), *Streptococcus* (Dramsi *et al*., 2006) and *Bacillus* (Budzik *et al*., 2007), as well as the sporulation of *Bacillus anthracis* (Marraffini and Schneewind, 2006).

Streptomyces coelicolor is a member of the Gram-positive actinomycetes, and is predicted to encode seven sortase enzymes (Pallen *et al*., 2001). Genome-wide analyses have estimated that between 9 and 17 sortase substrates are encoded by *S. coelicolor* (Pallen *et al*., 2001; Boekhorst *et al*., 2005). These predicted substrates all have an N-terminal signal peptide, as would be expected of proteins destined for secretion to the cell surface, but in place of a C-terminal LPXTG-containing sorting signal, which is the norm for most substrates anchored by SrtA-like 'housekeeping' sortases, *S. coelicolor* substrates have an LAXTG pentapeptide motif (Pallen *et al*., 2001). This proline to alanine substitution at the second position appears to be confined to the actinomycetes, with substrates of the housekeeping sortase in *Corynebacterium* also having LAXTG-containing sorting signals (Mandlik *et al*., 2008b). Within the streptomycetes, only four putative sortase substrates have been characterized to any extent. CbpC is a surface-localized carbohydrate-binding protein, and this localization requires an intact sorting signal (Walter and Schrempf, 2008); the biological function of CbpC remains to be determined. The other three characterized substrates (ChpA, B and C) belong to the 'chaplin' family of cell surface proteins, which collectively facilitate cellular differentiation during growth on solid media (Claessen *et al*., 2003; Elliot *et al*., 2003; Capstick *et al*., 2007).

S. coelicolor has a complex developmental cycle that initiates with spore germination and hyphal outgrowth to form a filamentous vegetative mycelium. From these vegetative cells emerge 'aerial hyphae', which confer a fuzzy white appearance to the growing colony. These aerial hyphae ultimately metamorphose into chains of grey-pigmented dormant exospores (Flärdh and Buttner, 2009). The transition between vegetative growth and aerial hypha formation requires the activity of the eight chaplin proteins. The chaplins are thought to provide a hydrophobic coating to the aerial structures (hyphae and spores), and have surfactant activity that likely facilitates the raising of aerial hyphae from the aqueous colony surface (Claessen *et al*., 2003). During growth in standing liquid culture, they also contribute to the formation of fimbriae (de Jong *et al*., 2009a). The chaplins are divided into two groups: three long chaplins (ChpA–C) which are predicted to have C-terminal sorting signals, and five amyloid-forming short chaplins (ChpD–H) (Claessen *et al*., 2003; Elliot *et al*., 2003). There appears to be considerable functional redundancy shared by both the long and short chaplins: at least four chaplin genes (two long and two short) must be deleted before any developmental defects are observed (Claessen *et al*., 2003; Elliot *et al*., 2003). The current model of chaplin assembly on the cell surfaces involves the long chaplins being covalently anchored to the cell wall by a sortase enzyme, and the short chaplins (which have no obvious anchoring sequence) self-assembling between long chaplin anchors (Di Berardo *et al*., 2008). The chaplin oligomers are then proposed to be organized into a higher-order 'rodlet' ultrastructure by a second class of secreted proteins known as the rodlins (Claessen *et al*., 2004). We hypothesized that a sortase mutant would have developmental defects stemming from the loss of long chaplin anchoring to the cell wall, and correspondingly, an inability to effectively tether the short chaplins to the aerial surfaces.

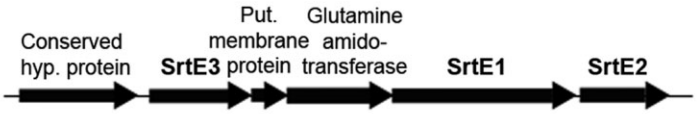
Here, we provide experimental evidence showing that two sortases are required for aerial development in *S. coelicolor*, and that these enzymes mediate long chaplin anchoring. Unexpectedly, we find that sortase mutants fail to transcribe key developmental genes, and discover that the long chaplins are not critical for short chaplin surface association, leading us to revise our model of aerial hyphae formation and chaplin surface assembly.

Results

Bioinformatic prediction of sortases and their substrates in S. coelicolor

Previous bioinformatic analyses of bacterial genomes have suggested that there are seven sortase genes encoded within the *S. coelicolor* chromosome (Pallen *et al*., 2001; Comfort and Clubb, 2004). Sortase enzymes are typically grouped into one of five categories on the basis of sequence homology. Two of the *S. coelicolor* sortases (SCO3849 and SCO3850) are classified as 'SrtE'-type sortases, along with sortases from other sporulating bacteria and actinomycetes (Spirig *et al*., 2011). The remaining five sortases are grouped in 'Class F' due to their divergent sequences and unknown functions (Comfort and Clubb, 2004; Spirig *et al*., 2011). Notably, these divergent sortases are all expressed from chromosomal positions flanked by potential substrate-encoding genes (Fig. S1), specifying proteins with an N-terminal signal peptide and a C-terminus having sorting signal-like characteristics (a hydrophobic stretch followed by several positively charged residues), although lacking an obvious pentapeptide recognition/cleavage motif (i.e. devoid of LAXTG, LPXTG or NPQTN motifs). These flanking substrates appear to be conserved in other *Streptomyces* species that encode the associated sortase gene (Fig. S1).

Housekeeping sortases tend to be encoded at sites disparate from that of their substrate-encoding genes



S. coelicolor	*SCO3853*	*X*	*3852*	*3851*	*3850*	*3849*
S. avermitilis	*SAV4332*	*4333*	*4334*	*4335*	*4336*	*4337*
S. griseus	*SGR3719*	*3720*	*3721*	*3722*	*3723*	*3724*
S. scabies	*SCAB45621*	*45611*	*45601*	*45591*	*45581*	*45571*
S. venezuelae	*SVEN3639*	*3637*	*3636*	*3635*	*3634*	*3633*

Fig. 1. Organization of the genetic region surrounding *srtE1* (*SCO3850*) and *srtE2* (*SCO3849*) in *S. coelicolor*, *S. avermitilis*, *S. griseus, S. scabies* and *S. venezuelae*. Gene designations are indicated below the schematic diagram, while predicted gene products are indicated above.

(Marraffini *et al*., 2006), and notably, neither of the two SrtE-encoding sortase genes (*SCO3849* and *SCO3850*) is flanked by obvious substrate-encoding genes. Thus, we predict that one or both of these enzymes function as the major housekeeping sortase in *S. coelicolor*. We propose these genes be renamed *srtE1* (*SCO3850*) and *srtE2* (*SCO3849*) to reflect their prior categorization and their predicted recognition of a distinct pentapeptide motif (LAXTG) compared with other SrtA-like housekeeping sortases (LPXTG). *srtE1* and *srtE2* are adjacent to each other in all *Streptomyces* genomes sequenced to date. In *S. coelicolor*, they appear to be the final two genes of an extended five gene operon; however, in other streptomycetes, this operon includes an additional (third) sortase gene, which we have termed *srtE3* (Fig. 1). SrtE3 is more similar in sequence to SrtE1 than SrtE2; however, it lacks the N-terminal extension that is exclusive to SrtE1 proteins (Fig. S2).

Developmental effects of srtE1 *and* srtE2 *knockouts*

Our first step in investigating the biological role of the sortases in *S. coelicolor* was to create null mutations of each gene. Knocking out any of the five 'Class F' sortase genes had no obvious phenotypic consequences when compared with their wild-type parent (Y.G. Jung and M.J. Buttner, pers. comm.), while replacement of either *srtE1* or *srtE2* with an apramycin resistance cassette had adverse developmental effects. On mannitol-soy flour (MS) agar medium (poor carbon source), the *srtE1* mutant (Table S1) had a significant developmental delay: it was 'bald' at day two, initiated aerial hyphae formation on day three, but was virtually indistinguishable from a wild-type strain by day six (Fig. 2A). In contrast, the *srtE2* mutant (Table S1) looked very similar to its wild-type parent at all times (Fig. 2A). Neither single mutant strain had any obvious developmental defects when grown on R2YE (rich, glucose-containing) agar medium (Fig. 2A). To eliminate any polar effects on *srtE2* stemming from the knockout of *srtE1*, the antibiotic resistance cassette was removed from *srtE1*, creating an in-frame deletion; the mutant phenotype was very similar to that of the cassette-containing strain (Fig. 2A).

A double *srtE1/E2* mutant was also constructed and its phenotype was more severe than that of either single mutant. Growth for 6 days on MS agar revealed a white colony phenotype, suggesting an inability to form mature spores, compared with the bald colony phenotype exhibited by the eightfold (Δ*chp*) chaplin knockout strain (Fig. 2A). This was supported by scanning electron micrographs of the *srtE1/E2* mutant, which revealed no detectable spore chains following growth for 5–7 days on MS agar (Fig. 2B). On R2YE agar medium, the mutant was slower to initiate aerial hyphae formation than the wild type (like the Δ*chp* strain) (Fig. 2A), but even after 10 days of incubation, no spore chains could be detected by coverslip impression (Fig. S3). The mutant strain could be fully complemented with a DNA fragment that contained only the two sortase genes flanked by additional upstream (133 bp) and downstream (224 bp) sequences (Fig. 2C). This suggested that *srtE1* and *srtE2* were transcribed independently of the three upstream genes, despite an apparent translational coupling of *srtE1* with the gene immediately upstream (*SCO3851*). Complementation of the double mutant with either *srtE1* or *srtE2* resulted in strains with phenotypes reminiscent of the single mutants (Fig. 2C).

SrtE1 has an extended cytoplasmic N-terminus not associated with other sortase enzymes

Given that SrtE1 had an N-terminal extension not found in any other characterized sortase enzymes, we wondered whether the start codon had been mis-annotated. The annotated start codon for *srtE1* overlapped the stop codon of the upstream gene, suggesting that these two genes were translationally coupled. This organization seemed inconsistent with our observation that the *srtE1/E2* mutant could be complemented with a DNA fragment encompassing only these two genes, as this suggested that they were expressed independently of the upstream genes. We identified an alternative start codon

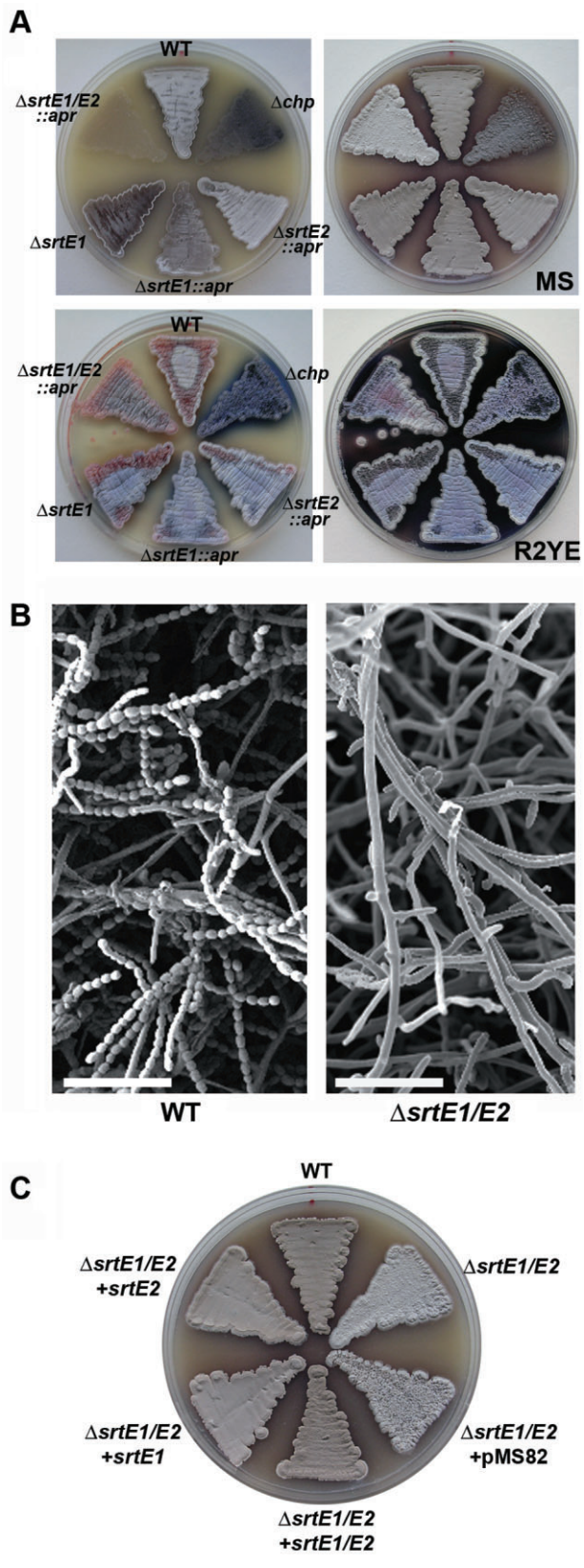


Fig. 2. Phenotypic comparison of sortase mutant strains with chaplin-deficient (Δ*chp*) and wild-type *S. coelicolor*.
A. Colony morphology of a chaplin-deficient strain (Δ*chp*), individual sortase mutant strains (Δ*srtE1::apr*, Δ*srtE1* and Δ*srtE2::apr*), a double sortase mutant (Δ*srtE1/E2::apr*) and their wild-type parent strain M600 (WT). Strains were grown on MS (top panels) or R2YE (bottom panels) agar medium for 2 days (left panels) or 6 days (right panels).
B. Scanning electron micrographs of M600 wild type (left) and the Δ*srtE1/E2* double mutant (right) after growth on MS agar medium for 5 days. White bars: 10 μm.
C. Complementation of the Δ*srtE1/E2* mutant strain with *srtE1, srtE2* or *srtE1/E2* together, on the integrating plasmid vector pMS82. Strains were grown on MS agar medium for 5 days.

just upstream of the N-terminal transmembrane region, in a position consistent with the start site of other sortases (Fig. S2). Using site-directed mutagenesis, we altered both the annotated start site and the alternative start codon of *srtE1*. We then tested the activity of these different *srtE1* constructs in the *srtE1/E2* mutant to see whether they could restore sporulation to the level observed following introduction of wild-type *srtE1* (Fig. 2C). The construct lacking the alternative start codon effectively stimulated sporulation of the *srtE1/E2* mutant; however, no sporulation was observed when the annotated start codon was mutagenized (GTG to GAG) (Fig. S4). This suggested that the start site for *srtE1* was correctly annotated, and that it did indeed overlap the stop codon of *SCO3851*. Furthermore, it suggested that there may be a promoter responsible for driving the expression of *srtE1* and *srtE2* within *SCO3851*.

srtE1 *and* srtE2 *are transcribed independently of the upstream gene,* SCO3851

We set about investigating the expression of *srtE1* and *srtE2* relative to each other, and to the upstream gene *SCO3851*, using semi-quantitative RT-PCR. We found *SCO3851* was expressed constitutively over a 96 h time-course, and at a relatively high level compared with *srtE1* and *srtE2* (Fig. 3). The two sortase genes appear to be co-transcribed, and were expressed throughout the first 72 h of growth (Fig. 3). To determine whether *srtE1* was co-transcribed with *SCO3851*, or whether it was expressed from a promoter located within *SCO3851*, we took advantage of the fact that the DNA fragment used to complement our *srtE1/E2* mutant strain extended 133 bp upstream of the *srtE1* start site. We generated cDNA from a primer within the *srtE1*-coding sequence, and used this as template to amplify two different products: one extending beyond the 133 bp end of our complementing clone [fragment (ii) in Fig. 3A], allowing us to identify any readthrough transcription from *SCO3851*, and the other extending just upstream of the *srtE1* start site [at the 3′ end of *SCO3851*; fragment (iii) in Fig. 3A]. We observed no detectable

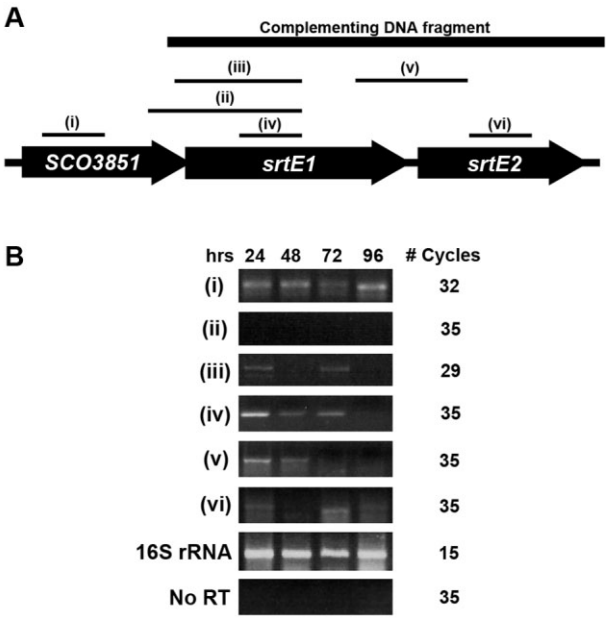


Fig. 3. Transcriptional analysis of *SCO3851, srtE1* and *srtE2*.
A. Schematic diagram indicating the three genes under investigation, and the position of products resulting from reverse transcription (RT) PCR [labelled (i) to (vi), and corresponding to those reactions shown in (B)], relative to both sortase genes and the DNA fragment used to complement the *srtE1/E2* mutant phenotype.
B. Semi-quantitative RT-PCR of transcripts within *SCO3851* (i), *srtE1* (iv) and *srtE2* (vi), as well as those extending between genes (ii, iii and v). The number of PCR amplification cycles was optimized to ensure products were in the linear amplification range, and that no products were observed in the negative control for each reaction. 16S rRNA served as a positive control for RNA levels, and for successful reverse transcription and PCR amplification.

product when amplifying beyond the end of the complementing DNA fragment [product (ii)], suggesting that there was no appreciable transcriptional readthrough from *SCO3851* into *srtE1*, but we did detect significant products (even with reduced cycle numbers) when amplifying the fragment extending to just upstream of *srtE1* [product (iii)] (Fig. 3B). This supported the proposal that *srtE1* and *srtE2* were transcribed independently of the upstream genes, from a promoter located within *SCO3851*.

SrtE1 and SrtE2 cleave LAXTG motif peptides

In most Gram-positive bacteria, the substrates of SrtA-like housekeeping sortases have a distinct LPXTG-containing C-terminal sorting signal. In *Streptomyces* and other actinomycetes, however, predicted substrates have a C-terminal LAXTG-containing motif. To investigate the enzymatic activity and pentapeptide motif-specificity of SrtE1 and SrtE2, we overexpressed and purified their extracellular domains (engineered to have an N-terminal 6× His tag), along with active-site mutant variants (Cys → Ala) for use in *in vitro* cleavage assays. Full-length versions of these active-site mutants were non-functional *in vivo*, as they failed to complement their respective null mutant phenotypes (Fig. S5). As a positive control, the well-characterized SrtA from *S. aureus* was also overexpressed and purified as an N-terminally 6× His-tagged fusion protein. Peptide cleavage capabilities and substrate specificity were assessed using a fluorescent reporter assay. Short (nine-amino-acid) peptide substrates were N-terminally labelled with a 2-aminobenzyl fluorophore, while a fluorescence-quenching dinitrophenyl group was affixed to their C-terminus. Fluorescence would be inhibited when fluorophore and quencher are in close proximity (on the same peptide), but would increase when the intervening peptide sequence is cleaved, separating fluorophore from quencher. Higher fluorescence levels in the presence of a sortase enzyme can thus be correlated with peptide cleavage.

We investigated the cleavage activity of the different sortase enzymes using peptides having four different core pentapeptide sequences: LAETG, LAHTG, LPETG and NPQTN. The first two sequences represent the predicted sortase recognition motifs for the three long chaplins from *S. coelicolor* (LAETG for ChpA and ChpC; LAHTG for ChpB). LPETG is an effective substrate for cleavage by the *S. aureus* SrtA enzyme (Mazmanian *et al.*, 1999) and served as a positive control for the assay, while NPQTN is not recognized by SrtA, instead being a substrate of SrtB from *S. aureus* (Mazmanian *et al.*, 2002). As expected, increased fluorescence was observed when SrtA was incubated with the LPETG sequence, but was not observed following incubation of SrtA with any of the other three peptide sequences (Fig. 4A). SrtE1 and SrtE2 could cleave both chaplin-associated sequences (LAETG/LAHTG), but did not effectively cleave either the LPETG peptide sequence or the NPQTN SrtB-specific sequence. As predicted, the active-site Cys mutant variants of SrtE1 and SrtE2 had significantly reduced cleavage activity ($P < 0.01$) relative to their wild-type counterparts.

To further examine the cleavage of these peptide substrates, the cleavage products were analysed by liquid chromatography-mass spectrometry (LC-MS). SrtA cleaved its LPETG substrate exclusively between the Thr and Gly residues (Fig. 4B), as has been observed previously (Mazmanian *et al.*, 1999). In contrast, while SrtE1 and SrtE2 also cleaved between the Thr and Gly residues of the LAXTG pentapeptide motif, products corresponding to cleavage between the second and third residues (Ala and His/Glu) were typically more abundant (Fig. 4B; Fig. S6). None of these products was detected for either of the active-site mutant enzymes (Fig. S6). This suggested that SrtE1 and SrtE2 were more flexible in their substrate cleavage than the prototypical SrtA enzyme, at least *in vitro*.

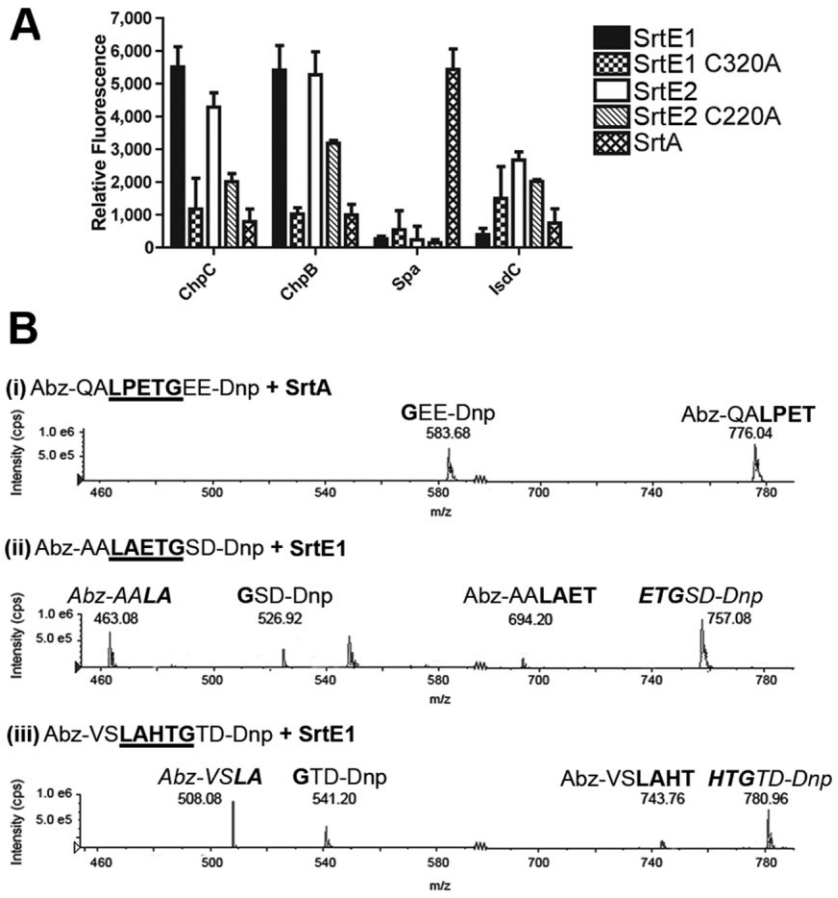


Fig. 4. Sortase cleavage assay. Both wild-type and active-site mutant variants of SrtE1 and SrtE2, as well as SrtA from *S. aureus*, were incubated with four different peptides, each containing a different core pentapeptide motif: LAETG (from ChpC, a putative SrtE1 and SrtE2 substrate), LAHTG (from ChpB, a putative SrtE1 and SrtE2 substrate), LPETG (from Spa – *S. aureus* protein A – a known SrtA substrate) and NPQTN (from IsdC, a known SrtB substrate). Each peptide had a fluorescent molecule (Abz) affixed to its N-terminus, and a quencher (Dnp) attached to its C-terminus. Cleavage of the intervening sequence led to an increase in fluorescence, shown in (A) as relative fluorescence, expressed in arbitrary units. Results shown are the average of at least eight assays, with each experiment performed in duplicate using four independent enzyme preparations.
B. MALDI-ToF mass spectrometry of the cleavage products resulting from peptide incubation with: (i) SrtA (LPETG), (ii) SrtE1 (LAETG) and (iii) SrtE1 (LAHTG). The pentapeptide sorting motif is indicated in bold in all instances. Products resulting from cleavage between the fourth and fifth residue in the pentapeptide motif (between the Thr and Gly) are indicated in regular font, while those products resulting from cleavage between the second and third residues (between the Ala and Glu/His) are shown in italics.

The long chaplins are substrates for SrtE1 and SrtE2

The results of the peptide cleavage assay suggested that the long chaplins (ChpA, B and C) were likely substrates for SrtE1 and SrtE2. Given this, we sought to determine the *in vivo* relevance of these observations, and followed the cell surface anchoring of a FLAG-tagged ChpC variant in different sortase mutant strains, where the FLAG tag was introduced just before the C-terminal sorting signal of ChpC (Fig. 5A). Given the low transcription levels of the long chaplin genes (M.A. Elliot, unpubl. data), we cloned the *chpC-FLAG* construct behind the constitutive *ermE** promoter on an integrating plasmid vector, and introduced this construct into a 7× chaplin mutant strain (J3149A), as well as into J3149A containing a *srtE1* knockout, a *srtE2* knockout or a *srtE1/E2* double knockout. Using J3149A (expressing only ChpE) was intended to simplify the ChpC localization studies: the long chaplins are predicted to act as anchors for the surface localized, insoluble short chaplin polymers, and such an association could adversely affect our ability to detect ChpC using polyacrylamide gel electrophoresis and immunoblotting. To further facilitate these studies, we conducted these experiments using liquid-grown cultures to ensure effective cell fractionation when examining ChpC localization.

In strains expressing both SrtE1 and SrtE2, ChpC-FLAG was either present in the culture supernatant or associated with the cell wall, while in the *srtE1/E2* double mutant, cell wall-associated ChpC-FLAG was never observed (Fig. 5B), even upon extended exposure of the immunoblot (data not shown). ChpC-FLAG levels in the individual sortase mutants varied between different protein preparations (e.g. levels in a *srtE1* strain were sometimes equivalent to wild type, and sometimes less, although they were never more abundant). Overall trends were, however, reproducible: wall-associated ChpC-FLAG was always less abundant in a *srtE2* mutant than in a *srtE1* strain (Fig. 5B). Occasionally a larger protein band could be detected, as seen in the lane corresponding to the culture supernatant of the *srtE1/E2* mutant strain in Fig. 5B. This likely represents full-length ChpC-FLAG which was presumably released into the supernatant through cell lysis, as it could also be detected in the protoplast fraction of the *srtE1/E2* mutant strain (Fig. 5B) and in other protoplast fractions upon immunoblot overexposure (data not shown), but was never detected in the wall fraction or in the

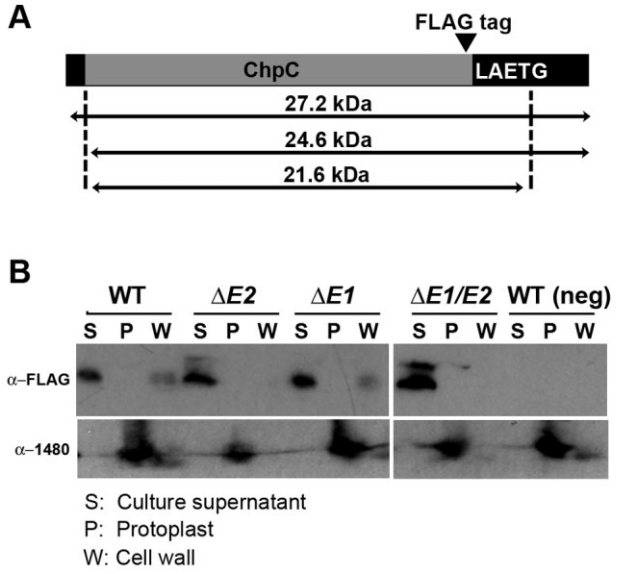


Fig. 5. *In vivo* analysis of ChpC-FLAG cell wall attachment by SrtE1 and SrtE2.
A. A FLAG tag was introduced into the ChpC sequence, just before the LAETG sorting signal. ChpC has an N-terminal signal peptide and a C-terminal sorting signal (both indicated in black). Full-length (unmodified) ChpC-FLAG would have a molecular weight of 27.2 kDa, while removal of its signal peptide would result in a product of 24.6 kDa, and removal of the C-terminal sorting signal would further reduce its molecular weight to 21.6 kDa.
B. Immunoblot of culture supernatant (S), protoplast (P) and cell wall (W) fractions of liquid-grown *S. coelicolor* cultures. Strains tested included a chaplin mutant (J3149A) expressing wild-type *srtE1* and *srtE2* (WT), J3149A lacking *srtE2* (Δ*E2*), J3149A lacking *srtE1* (Δ*E1*) and J3149A lacking both *srtE1* and *srtE2* (Δ*E1/E2*). Each of these strains also contained an integrating plasmid vector (pIJ82) bearing *chpC-FLAG* cloned behind a constitutive (*ermE**) promoter. As a negative control [WT(neg)], we used the WT strain carrying only pIJ82 (lacking *chpC-FLAG*). Top panels show blots probed with anti-FLAG antibodies, while the bottom panels show the same blots probed with anti-SCO1480 antibodies (where SCO1480 is a cytoplasmic protein) as a control for effective cell fractionation.

negative control (strain lacking ChpC-FLAG; Fig. 5B). Full-length (unprocessed) ChpC-FLAG has an expected molecular weight of 27.2 kDa, compared with fully processed ChpC-FLAG which has a predicted molecular weight of 21.6 kDa. As a control for appropriate cell fractionation, blots were probed with an antibody specific for the cytoplasmic protein SCO1480 (Fig. 5B). Taken together, these results support our *in vitro* data, and indicate that SrtE2, and to a lesser extent SrtE1, are required for the anchoring of ChpC-FLAG to the cell wall.

Sortase mutants lack a rodlet ultrastructure and are defective in anchoring several short chaplins

The current model for chaplin assembly on the surface of aerial hyphae and spores involves the oligomerization of short chaplin fibres between cell wall-anchored long chaplins. Given that a *srtE1/E2* mutant was unable to affix ChpC-FLAG to its cell wall, we wondered whether the striking developmental defects of this strain could be attributed to inappropriate localization/assembly of the short chaplins. Using MALDI-ToF mass spectrometry, we compared the short chaplin profile of the *srtE1/E2* mutant after 6 days of growth on MS agar plates with that of a wild-type strain. The sortase mutant consistently exhibited an altered profile relative to the wild type: the peaks corresponding to ChpD and ChpF were dramatically decreased relative to ChpE and ChpH (the ChpG peak is always very small and was not detected in this work) (Fig. 6A). We further probed the surface structure of the *srtE1/E2* mutant strain using high-resolution scanning electron microscopy, and found it to have an aerial mycelium that was largely devoid of both spore chains and any semblance of rodlet ultrastructure (Fig. 6B), suggesting that this mutant was indeed defective in short chaplin anchoring and organization.

A long chaplin mutant has a subtle developmental defect and a normal short chaplin profile

To test whether the defect in short chaplin localization observed for the *srtE1/E2* mutant was due to loss of effective long chaplin anchoring, we set about constructing a 'long chaplin mutant' (Δ*chpABC*), and compared its phenotype with that of the *srtE1/E2* mutant. The Δ*chpABC* mutant strain was delayed (~ 24 h) in its ability to raise aerial hyphae relative to the wild-type strain when grown on MS agar medium, suggesting an early developmental

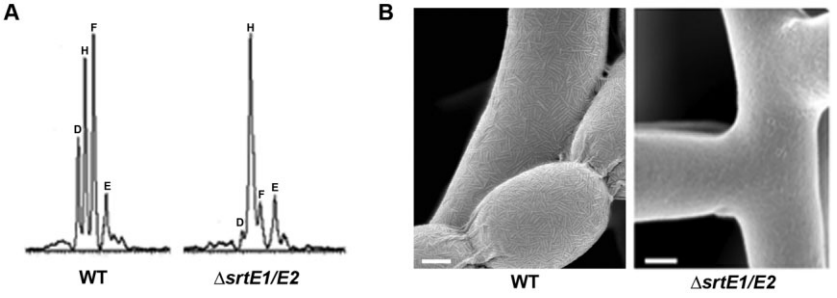


Fig. 6. Examination of short chaplins on the surface of M600 wild-type (WT) and sortase mutant (Δ*srtE1/E2*) strains.
A. MALDI-ToF mass spectrometry of short chaplins extracted from the surface of 6-day MS agar-grown cultures.
B. High-resolution scanning electron micrographs of the surfaces of a wild-type (left) and Δ*srtE1/E2* mutant (right) strain after 5 days of growth on MS agar medium. White bars: 200 nm.

role for the long chaplins; however, the Δ*chpABC* strain generally exhibited a far more robust aerial mycelium than either the *srtE1/E2* or Δ*chp* (8×) mutant strain (Fig. 7A). We probed the short chaplin profile of the Δ*chpABC* strain after six days of growth using mass spectrometry, and found it effectively mirrored that of the wild type, suggesting that in contrast to existing models, the long chaplins were not required for short chaplin association with the cell surface (Fig. 7B). We examined the long chaplin mutant for evidence of surface ultrastructure, and found the spores to possess a clear rodlet architecture (Fig. 7C). Intriguingly however, individual rodlets were generally longer in the Δ*chpABC* mutant than in the wild-type strain (Fig. 7D), having an average length of 172 nm compared with 109 nm for the wild type [as determined using ImageJ; $n = 207$ (wild type) and 369 (mutant)]. Thus, it appears that while the long chaplins are not necessary for localization of the short chaplins later in development, they likely play a role in initiating aerial hyphae formation, and contribute to the overall surface ultrastructure. Furthermore, it implies that the defect in short chaplin organization seen in the sortase mutant cannot be due to loss of the long chaplins.

Sortase mutants show reduced expression of the rdlA *and* nepA *genes, and several short chaplin genes*

As the long chaplins are not required for attaching the short chaplin polymers to the aerial surfaces, we wondered whether the reduced levels of ChpD and ChpF observed for the *srtE1/E2* mutant were the result of mislocalization, or whether these chaplin proteins were not expressed at wild-type levels. We compared the transcription of *chpH, chpD* and *chpF* in wild type, Δ*srtE1/E2* and Δ*chpABC* mutant strains during growth on MS agar (a condition where the Δ*srtE1/E2* mutant fails to form spore chains, and has unusually branched aerial hyphae; Figs 2B and 6B). We found *chpH* expression to be similar in all three strains, whereas in the *srtE1/E2* mutant, *chpD* transcript accumulation was significantly delayed and *chpF* expression was virtually undetectable (Fig. 8). Previous transcription profiling experiments showed that *chpD* and *chpF* were most highly expressed during aerial hyphae formation (Elliot *et al*., 2003), and thus we decided to investigate whether aerial hyphae-specific gene expression was compromised in the *srtE1/E2* mutant. We examined the expression of *rdlA*, which encodes one of the two rodlin proteins that contribute to rodlet formation on aerial surfaces (Claessen *et al*., 2002), and *nepA*, which encodes a small secreted protein that influences development (Dalton *et al*., 2007; de Jong *et al*., 2009b), both of which are expressed only following the initiation of aerial hyphae formation. These two genes were not expressed at detectable levels in the *srtE1/E2* mutant, whereas they were expressed at a time

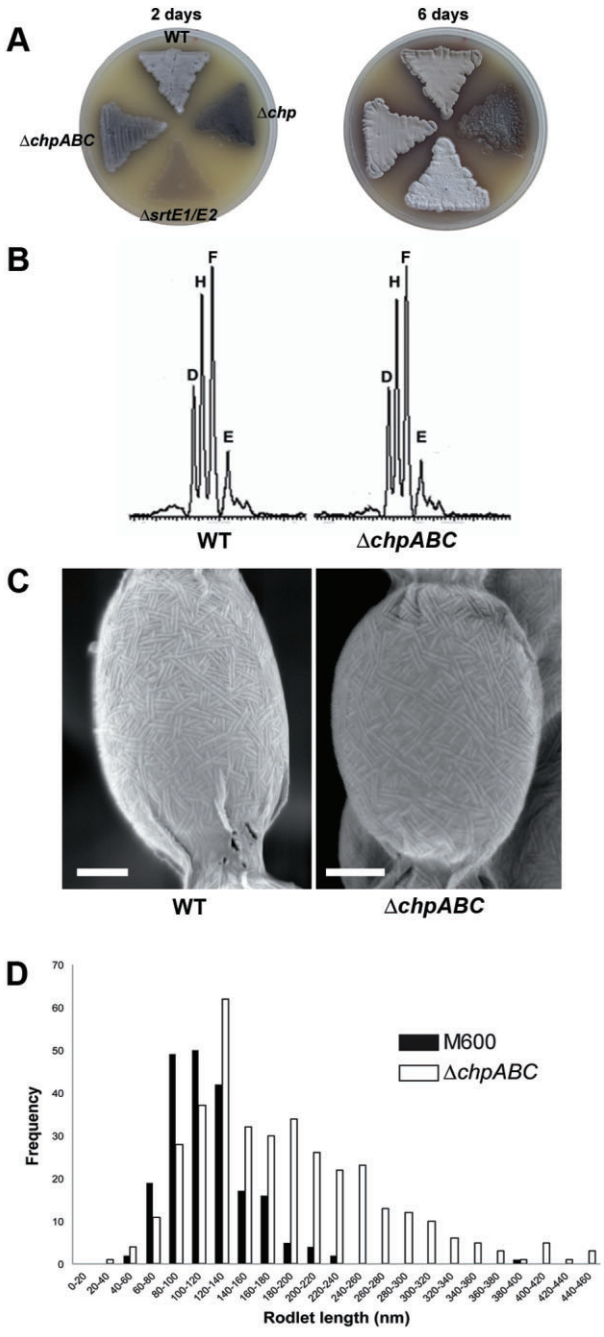


Fig. 7. Phenotypic analysis of the long chaplin mutant Δ*chpABC*.
A. Colony morphology of M600 wild type (WT), eightfold chaplin mutant (Δ*chp*), long chaplin mutant (Δ*chpABC*) and double sortase mutant (Δ*srtE1/E2*) after 2 days (left) or 6 days (right) growth on MS agar medium.
B. MALDI-ToF mass spectrometry of short chaplins, extracted from the surface of M600 wild-type (WT) and long chaplin mutant (Δ*chpABC*) strains after 6 days of growth on MS agar medium.
C. High-resolution scanning electron micrographs of spore rodlet ultrastructure from M600 wild-type (WT) and long chaplin mutant (Δ*chpABC*) strains after 5 days of growth on MS agar medium. White bars: 200 nm.
D. Distribution of rodlet lengths (nm) in M600 wild-type (black bars; $n = 207$) and Δ*chpABC* mutant (white bars; $n = 369$) strains, as determined using ImageJ.

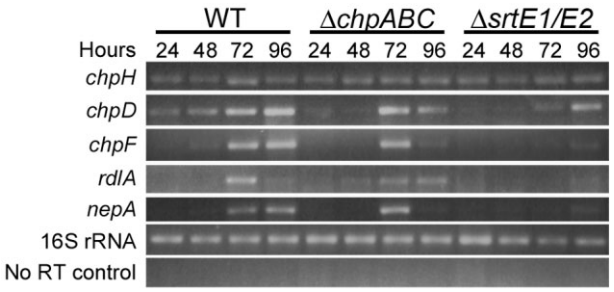


Fig. 8. Semi-quantitative RT-PCR using RNA extracted from wild-type strain M600 (WT), the long chaplin mutant (Δ*chpABC*) and a double sortase mutant (Δ*srtE1/E2*) grown for 24, 48, 72 and 96 h on MS agar medium. The number of PCR amplification cycles was optimized for each transcript (29 cycles for *rdlA, chpD*, *chpF* and *chpH*; 30 cycles for *nepA*; 15 cycles for 16S rRNA). 16S rRNA served as a positive control for RNA levels, and for successful reverse transcription and PCR amplification.

consistent with aerial hyphae formation and sporulation in both the wild-type and Δ*chpABC* mutant strains. (Fig. 8), suggesting that loss of the two sortase enzymes leads to a developmental block that adversely affects aerial hyphae-specific gene expression.

Developmental roles for other sortase substrates

In an attempt to determine the sortase substrate(s) responsible for the developmental defects of the *srtE1/E2* mutant, we examined the remaining 11 (non-chaplin) predicted sortase substrates (Comfort and Clubb, 2004; Boekhorst *et al*., 2005) (Table S2). We hypothesized that any substrate having a significant role in development may be broadly conserved in the streptomycetes. We selected two well-conserved (in *S. coelicolor, S. griseus, S. avermitilis, S. venezuelae* and *S. scabies*) substrate-encoding genes for investigation: *SCO1734* and *SCO2971*. *SCO2971* encodes an uncharacterized secreted protein with an unusual repeating sequence (where the repeated sequence itself is not conserved between species, but the repeat organization is shared by all species), while *SCO1734* encodes the cellulose-binding protein CbpC (Walter and Schrempf, 2008). Each gene was knocked out, and the resulting mutant phenotypes were assessed. Neither knockout had any obvious effect on colony development during growth on R2YE, MS or minimal agar media (data not shown), indicating that neither of these gene products is responsible for the sortase-mediated developmental block.

Discussion

Sortase enzymes are used by a wide variety of Gram-positive bacteria to modify their cell surfaces through covalent protein attachment. The resulting sortase-anchored proteins contribute to everything from pilus assembly and virulence, to iron uptake and sporulation. Here, we expand the biological role of sortases to include aerial development in *S. coelicolor*.

It is not unusual for bacteria to encode more than one sortase enzyme, but in these instances, they typically encode one 'housekeeping' sortase and one or more specialized sortases. A classic example of this is in *S. aureus*, where SrtA anchors a number of genetically disparate proteins containing an LPXTG sorting signal (many of which are involved in virulence and adhesion); in contrast, SrtB specifically targets substrates involved in iron acquisition, whose coding sequences are clustered with the *srtB* gene on the chromosome and have a sorting signal distinct from that of SrtA substrates (Mazmanian *et al*., 2002). *S. coelicolor* is unusual in that it appears to have two housekeeping sortases, along with five additional sortase enzymes that likely have more specialized activities. Interestingly, most other streptomycetes appear to encode a third housekeeping sortase in the same gene cluster as *srtE1* and *srtE2* in *S. coelicolor*. Multiple housekeeping sortases are not a universal property shared by the actinomycetes, but rather seem to be confined to the streptomycetes.

Housekeeping sortases – distinct roles for SrtE1 and SrtE2

We found the functions of SrtE1 and SrtE2 were not completely redundant, as the loss of either enzyme conferred a different developmental phenotype. The double sortase mutant phenotype was, however, far more severe than that of either single sortase mutant, suggesting that each enzyme must be able to at least partially compensate for the loss of the other. This functional redundancy was supported by our *in vitro* investigations, which revealed both enzymes to effectively cleave the long chaplin-sorting signals. When we examined the *in vivo* anchoring of ChpC, however, we discovered that SrtE1 and SrtE2 did not make equivalent contributions to its cell wall attachment, with the loss of SrtE2 having a much greater effect than the loss of SrtE1. An obvious explanation for this could be that SrtE2 is expressed at higher levels than SrtE1; however, our transcriptional analyses suggest this may not be the case. This apparent functional differentiation could instead be due to: (i) spatial segregation of the two sortase enzymes in the cell, leading to distinct substrate interactions, (ii) accessory proteins that provide substrate specificity, or (iii) specificity determinants encoded in the substrates themselves that result in preferential interaction with either of the two sortases. An obvious differentiating factor between SrtE1 and SrtE2 is the cytoplasmic N-terminal extension that is unique to SrtE1. In comparing the sequences of SrtE1 N-terminal extensions from different streptomycetes, there is only a small conserved region of approxi-

mately eight residues embedded in a longer sequence of 100–160 amino acids (Fig. S2). This conserved sequence corresponds to a positively charged patch, and it will be interesting to see whether this conserved region, and the N-terminus as a whole, has an important role in SrtE1 localization and activity.

Substrate specificity

All predicted housekeeping sortase substrates in the streptomycetes have an LAXTG-containing sorting signal (Table S2), in contrast to the classical LPXTG motif recognized by SrtA-like enzymes. This second position appears to be a major differentiator between SrtA and SrtE enzymes, at least *in vitro*, as SrtA cleaved LPXTG-containing peptides but not LAXTG-containing ones, while SrtE1 and SrtE2 exhibited opposing specificity. This may suggest that there are differences in the binding pocket of *Streptomyces* enzymes compared with their SrtA counterparts that result in either less effective binding of LPXTG-containing substrates or less effective cleavage of such a sequence. This is supported by an NMR co-structure of SrtA with a sorting signal analogue, which suggests that Ile^{182} and Ala^{118} interact with the proline of the LPXTG sorting motif (Suree *et al*., 2009); while the alanine residue is conserved in SrtE1 and SrtE2, the isoleucine is replaced with a threonine in all SrtE sequences (Fig. S2). Like SrtA, both SrtE1 and SrtE2 appeared to have little specificity for the third residue of the sorting signal, with motifs carrying either Glu or His residues at this position being recognized equally well.

Interestingly, the LAXTG-specificity of SrtE1 and SrtE2 was associated with unusual cleavage of the sorting signal. While some cleavage was observed between the Thr and Gly residues, a cleavage position common to all characterized sortases to date, a greater proportion of cleavage occurred immediately following the Ala residue, irrespective of the third position residue. The Ala–Pro substitution likely allows for a different association within the SrtE binding pocket. Whether this unusual cleavage is simply an *in vitro* phenomenon, or whether it occurs *in vivo* remains to be seen.

Sortases and S. coelicolor *development*

The double sortase mutant exhibited a strong defect in aerial development and sporulation. We had initially anticipated that these defects would be due to loss of the long chaplins, and a corresponding loss of short chaplin cell surface attachment. Surprisingly, this was not the case, and has led to our revising the existing model of chaplin-mediated aerial hyphae formation. A long chaplin mutant (Δ*chpABC*) exhibited an ~ 24 h delay in aerial hyphae formation, suggesting an early role in promoting aerial development. This delay was observed at a time when typically only two short chaplins are expressed (ChpE and ChpH; Elliot *et al*., 2003), and is consistent with previous work showing that the addition of a long chaplin-encoding gene (*chpC*) to a strain expressing only *chpE* and *chpH* resulted in enhanced aerial hyphae formation and sporulation (Di Berardo *et al*., 2008). Thus, we propose that the long chaplins initially have a role in promoting ChpE and ChpH assembly on the emerging aerial surfaces. In the absence of the long chaplins, this assembly is less efficient and requires short chaplin association with other, yet to be identified cell surface polymers. After 6 days, however, the Δ*chpABC* mutant had a normal complement of surface-localized short chaplins, and these were effectively organized into paired rodlets. Expression of the remaining short chaplins (ChpD, F and G) is upregulated once aerial hyphae formation initiates (Elliot *et al*., 2003), and thus we propose that at some threshold concentration of short chaplins, the long chaplins become dispensable and instead may have a more minor role in rodlet organization. The short chaplins are known to self-assemble into amyloid structures on the cell surface (Capstick *et al*., 2011; Sawyer *et al*., 2011). As amyloid formation can be readily 'seeded' by existing protein oligomers, one can envision a situation in which the initial (ChpE- and ChpH-based) short chaplin amyloids promote the incorporation of subsequently expressed short chaplins, in the absence of long chaplin assistance.

The *srtE1/E2* mutant retained ChpE and ChpH on its aerial surface, but did not exhibit any obvious surface ultrastructure. This could be readily explained by the lack of rodlin gene expression in this mutant (Fig. 8), as the rodlin proteins, together with the chaplins, are required for rodlet formation (Claessen *et al*., 2004). The general defect in aerial hyphae-specific gene expression observed for the double sortase mutant was completely unexpected. These findings implicate sortase activity in the control of a developmental checkpoint specific for aerial hyphae maturation, and suggest that one or more sortase substrates may trigger this developmental switch; such a role for sortase enzymes, and their substrates, is so far unprecedented. None of the predicted sortase substrates has known signalling or regulatory functions, so how this checkpoint is modulated remains to be determined. The existence of such a checkpoint has, however, been alluded to previously, on the basis of transcriptional profiling of a multiple chaplin mutant strain (Claessen *et al*., 2006; de Jong *et al*., 2009b), which, like the sortase mutant, fails to express aerial hyphae-specific genes.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Streptomyces coelicolor and *Escherichia coli* strains, as well as all plasmids used in this study are described in Table S1.

S. coelicolor strains were grown at 30°C on soy flour-mannitol (MS), R2YE, minimal or DNA agar media, or in a TSB–YEME liquid culture mixture, as described in Kieser *et al*. (2000). All *E. coli* strains were grown in LB or SOB liquid media or on LB agar plates at 37°C, except for BW25113, which was grown at 30°C.

Creation of sortase, long chaplin and sortase substrate knockout strains

Knockout of *srtE1, srtE2* and both genes together in both wild type (M600) and a 7× *chp* mutant background was accomplished using ReDirect technology (Gust *et al*., 2003). Briefly, each gene, or pair of genes, was replaced with an apramycin resistance cassette in cosmid StH69. These mutant cosmids were then conjugated from the non-methylating *E. coli* strain ET12567/pUZ8002 into either a wild-type or chaplin mutant strain. In each case, the apramycin resistance cassette was removed using the pUWL hyg FLP plasmid (a hygromycin-resistant derivative of pUWLFLP; Fedoryshyn *et al*., 2008), which carries the FLP recombinase gene under the control of the constitutive *ermE** promoter. This plasmid was conjugated into the mutant strains, selected with hygromycin, and colonies were screened for loss of apramycin resistance. All mutations were thoroughly checked by PCR (using both flanking and internal primer combinations; Table S3) to ensure either complete loss of the wild-type gene(s) (for initial knockouts) or loss of the antibiotic resistance cassette (when creating in-frame deletions). Complementation of the double sortase mutation was achieved by PCR-amplifying *srtE1* and *srtE2* together, using SCO3850p1 and SCO3849 end (Table S3), and cloning them into the SmaI site of pIJ2925 (Table S1). The two genes were then excised as a HindIII–KpnI fragment and cloned into the integrating vector pMS82, which had been digested with the same enzymes. The resulting construct was introduced into the mutant strain by conjugation from the non-methylating *E. coli* strain ET12567/pUZ8002 (Table S1). The same cloning and complementation strategy was used to generate individual SrtE1- and SrtE2-encoding constructs: *srtE1* was PCR-amplified using SCO3850p1 and SCO3850end, together with wild-type StH69 cosmid DNA as template, while *srtE2* was amplified using SCO3850p1 and SCO3849 end, from cosmid DNA in which *srtE1* had been deleted (replaced with an apramycin resistance cassette, followed by cassette excision) as template.

A similar procedure was used to knockout both *SCO1734* and *SCO2971*, only using cosmids StI11 and StE59 respectively.

To generate the 'long chaplin mutant' (Δ*chpABC*), the apramycin resistance cassette was first removed from J3144 (Δ*chpB::apr*; Table S1) as described in Elliot *et al*. (2003). *chpA* and *chpC* were then replaced in this strain, with apramycin (in cosmid StC46) and viomycin (in cosmid StI52) resistance cassettes, respectively, using the ReDirect technique described above.

Scanning electron and light microscopy

Wild-type, Δ*srtE1/E2* and Δ*chpABC* strains were grown for 4–6 days on MS agar medium before being examined by scanning electron microscopy. Microscopy was conducted as described previously (Di Berardo *et al*., 2008; Haiser *et al*., 2009). To evaluate sporulation by the wild-type and *srtE1/E2* mutant strains on R2YE agar medium, coverslip impressions were taken after 10 days, and were examined by light microscopy at 100× magnification.

Site-directed mutagenesis of srtE1 *and* srtE2

pMC131 (pIJ2925+*srtE1*; Table S1) was used as template for site-directed mutagenesis of the *srtE1* annotated start codon (GTG to GAG), an alternative start codon (V119A by altering GTG to GCG), and its active-site cysteine codon (C320A). In each case, complementary primers carrying the desired mutation in the centre (see Table S3) were used to amplify the entire pIJ2925+*srtE1* sequence, using PCR reactions detailed previously (Di Berardo *et al*., 2008). Template DNA was selectively degraded by DpnI digestion (which specifically cleaves methylated DNA). Three microlitres of the reaction were used for electroporation into XL1-Blue (Stratagene) cells, which were then plated on LB agar plates supplemented with ampicillin to select for positive transformants. Plasmid DNA was isolated from several representative transformants, and was sequenced to confirm the presence of the desired mutation and to ensure the integrity of the *srtE1* sequence. The mutagenized sequences were then excised from pIJ2925 using HindIII and KpnI and cloned into pMS82 before being conjugated into the *srtE1* knockout strain E200. A similar process was used to mutate the active-site cysteine residue of SrtE2, only using pMC132 (pIJ2925+*srtE2*; Table S1) as template DNA, and introducing the final mutant construct into the *srtE2* knockout strain E201.

Sortase overexpression and purification

Primers 3850 NdeI/3850 BamHI and 3849 NdeI/3849 BamHI (Table S3) were used to PCR-amplify the extracellular domains of *srtE1* and *srtE2*, respectively, from wild-type *S. coelicolor* M600 chromosomal DNA. Cysteine mutant variants of each were generated using pMC131a (*srtE1*) and pMC132a (*srtE2*) as template DNA for PCR. The resulting amplification products were digested with NdeI and BamHI, and cloned into pET15b, which had been digested with the same enzymes prior to being dephosphorylated. A similar strategy was used to amplify and clone the extracellular domain of *srtA*, only using primers SrtA NdeI/SrtA BamHI (Table S3) and *S. aureus* chromosomal DNA as template. All constructs were verified by sequencing. Each recombinant plasmid was introduced into chemically competent *E. coli* BL21 and BL21+Rosetta cells. Overnight cultures were used to inoculate 500 ml of LB medium supplemented with chloramphenicol, kanamycin and ampicillin, and these were grown at 37°C to an OD_{600} of 0.4 before adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) to induce protein overexpression. Cultures were then incubated for another 9.5 h at 26°C before cells were harvested by centrifugation. The resulting cell pellet was resuspended in 10 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) together with 1 mg ml^{-1} lysozyme and one cOmplete Mini EDTA-free protease inhibitor pellet (Roche), and incubated on ice for 30 min. The cell suspension was then sonicated on

ice for 6 × 10 s before being treated with RNase A (40 μg) and DNase I (20 U, Roche) for 15 min on ice. The suspension was centrifuged at 10 000 *g* for 30 min at 4°C, and the resulting supernatant was incubated with 1 ml of Ni-NTA slurry (Qiagen) for 90 min at 4°C, and then applied to a PolyPrep chromatography column (Bio-Rad). The column was washed with buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing increasing concentrations of imidazole (4 ml of 25 mM imidizole and 2 ml of 50 mM imidazole) before the protein was eluted using 500 μl each of 100, 200 and 500 mM imidazole-containing buffer. Purified proteins were applied to an Ultra Centricon centrifugal unit (Amicon), which was used to exchange the imidizole-containing buffer for protein storage buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT], after which glycerol was added to a final concentration of 10%. Purified proteins were separated on a 15% SDS polyacrylamide gel and were detected by staining with Coomassie Brilliant Blue.

Sortase cleavage assay

Sortase cleavage activity was monitored by incubating 100 μl reactions containing purified wild-type and mutant sortase proteins (10 μM or 50 μM final concentration), together with 50 μM of each modified peptide substrate in cleavage buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM DTT and 5 mM $CaCl_2$] for 18 h at 30°C. Peptide substrates (Biopeptide) included: AALAETGSD (ChpC sorting signal; putative SrtE1/E2 substrate), VSLAHTGTD (ChpB sorting signal; putative SrtE1/E2 substrate), QALPETGEE (SrtA substrate) and VENPQTNAG (SrtB substrate), with a 2-aminobenzoyl (Abz) fluorophore conjugated to the N-terminus, and a 2,4-dinitrophenyl (Dpn) quencher affixed to the C-terminus. Fluorescence was measured at 320 nm (excitation) and 420 nm (emission) for both the cleavage reactions and the peptides alone (with peptide alone fluorescence being subtracted from the overall reaction fluorescence). Reactions were conducted in duplicate using at least four independent sortase preparations.

Peptide cleavage analysis

LC-ESI-MS data were obtained using an Agilent 1100 Series LC system (Agilent Technologies Canada) and a QTRAP LC/MS/MS System (ABSciex). One hundred microlitres of the sortase cleavage reaction was subjected to reverse phase HPLC using a C_{18} column (3 μm, 120 Å, 4.6 × 100 mm; Dionex Corporation) at a flow rate of 1 ml min^{-1}, under the following conditions: isocratic 5% solvent B (0.05% formic acid in acetonitrile), 95% solvent A (0.05% formic acid in water) followed by a linear gradient to 97% B over 20 min.

Construction of ChpC-FLAG

An XhoI site was introduced into *chpC*, just upstream of the LAETG-encoding sequence using site-directed mutagenesis with primers USflagxho and DSflagxho (Table S3), together with pMC138 as template as described above. The resulting plasmid was confirmed by sequencing, and then digested with XhoI. A FLAG-tag-encoding cassette with XhoI-specific overhangs was then introduced into the XhoI-digested plasmid by ligation, and correct orientation of the FLAG cassette was confirmed by sequencing. The *chpC-FLAG* fusion was then amplified using M13R and chpC-up NdeI primers (Table S3), after which the resulting PCR product was digested with NdeI and XbaI, and cloned downstream of the constitutive *ermE** promoter in pMC500 digested with the same enzymes. This construct was checked by sequencing. The *ermE** promoter-*chpC-FLAG* fragment was then excised with BglII sites that flanked either side of the multiple cloning site, and cloned into pIJ82 digested with BamHI and dephosphorylated, to create pMC137. This resulting plasmid was conjugated into J3149A (7× *chp* mutant strain) and sortase mutant derivatives of this strain (E204, E203 and E202; Table S1).

Immunoblotting

Using fresh mycelium for inoculation, four *chpC-FLAG*-containing strains (J3149A, E204, E203 and E202), along with a negative control (J3149A containing pIJ82 alone), were grown for 16–20 h at 30°C in a 10 ml TSB–YEME mixture, after which the culture was scaled up by adding it to 40 ml of fresh TSB–YEME and grown overnight. The cells were pelleted by centrifugation at 1000 *g*, and the culture supernatant was transferred to a fresh tube. The cell pellet was washed twice with 10.3% sucrose and its wet cell weight was determined. The cells were then resuspended in ~ 1 ml of P-buffer containing lysozyme at a final concentration of 1 mg ml^{-1} and incubated at 30°C for 45 min, before being pipetted up and down three times and incubated for a further 15 min at 30°C. The cell mixture was then split in two. One half was sonicated for 3 × 10 s and represented the 'total' cell protein fraction. The other half was centrifuged at 1000 *g* for 7 min to separate protoplasts (pellet) from cell wall fragments (supernatant). The cell wall fraction was removed to a separate tube, and the protoplasts were resuspended in a volume equivalent to that of the cell wall fraction. The original culture supernatant was precipitated using trichloroacetic acid (TCA), and the resulting pellet was resuspended in a volume equivalent to that of the protoplasts and cell wall. Fifteen microlitres of each sample was then boiled together with an equivalent volume of sample buffer for 5 min before loading onto a 10% SDS-PAG. The separated proteins were transferred to PVDF membranes using a semi-dry Transblotter (Bio-Rad). The membrane was incubated for 1 h with 6% skim milk, overnight with the primary FLAG-tag-specific antibody (1/1500 dilution; Cell Signaling Technology), and for an hour with the secondary antibody (1/3000 dilution; Cell Signaling Technology). As a control for effective cell fractionation, membranes were also incubated with primary antibodies specific for the cytoplasmic protein SCO1480 (1/7000 dilution), followed by application of goat anti-rabbit secondary antibodies (1/3500 dilution; Cell Signalling Technology).

Chaplin extraction and MALDI-ToF mass spectrometry

The short chaplins were extracted from 6-day cultures grown on MS agar medium as described previously (Capstick *et al.*, 2007), and were analysed by MALDI-ToF mass spectrometry as outlined in Elliot *et al.* (2003).

RNA isolation and RT-PCR analysis

Wild-type *S. coelicolor* M600, Δ*chpABC* and Δ*srtE1/E2* mutant strains were grown on cellophane-coated MS agar medium for 24, 48, 72 and 96 h. RNA was harvested from these cultures as described in Swiercz *et al.*, (2008), before RNA concentration and integrity were determined using UV spectroscopy and agarose gel electrophoresis. Following this, reverse-transcription PCR (RT-PCR) was conducted as described previously (Hindra *et al.*, 2010). Briefly, 2 μg of RNA was used as template for reverse transcription using gene-specific primers (see Table S3) and Superscript III (Invitrogen), according to the manufacturer's instructions. The resulting cDNA then served as template for PCR amplification using *Taq* DNA polymerase and the gene-specific primers described in Table S3. The number of PCR cycles was optimized to ensure that products were assessed in the linear amplification range and that there were no products detected in the equivalent 'no RT' (RNA that had not been subject to reverse transcription) negative control [*chpH, chpD, chpF* and *rdlA* (29 cycles); *nepA* (30 cycles); *SCO3851* (32 cycles); *SCO3851-srtE1* (extended), *srtE1, srtE2, srtE1-E2* (35 cycles); *SCO3851-srtE1* (3′ end of *SCO3851*) (29 cycles)]. 16S rDNA was amplified (15 cycles) as a positive control. RT-PCR reactions were conducted at least four times, using two independent RNA samples.

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