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Function and Redundancy of the Chaplin Cell Surface Proteins in Aerial Hypha Formation, Rodlet Assembly, and Viability in *Streptomyces coelicolor*▽

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The chaplins are a family of eight secreted proteins that are critical for raising aerial hyphae in *Streptomyces coelicolor*. These eight chaplins can be separated into two main groups: the long chaplins (ChpA to -C) and the short chaplins (ChpD to -H). The short chaplins can be further subdivided on the basis of their abilities to form intramolecular disulfide bonds: ChpD, -F, -G, and -H contain two Cys residues, while ChpE has none. A "minimal chaplin strain" containing only *chpC*, *chpE*, and *chpH* was constructed and was found to raise a substantial aerial mycelium. This strain was used to examine the roles of specific chaplins. Within this strain, the Cys-containing ChpH was identified as the major polymerization unit contributing to aerial hypha formation and assembly of an intricate rodlet ultrastructure on the aerial surfaces, and the two Cys residues were determined to be critical for its function. ChpC augmented aerial hypha formation and rodlet assembly, likely by anchoring the short chaplins to the cell surface, while ChpE was essential for the viability of wild-type *S. coelicolor*. Interestingly, the lethal effects of a *chpE* null mutation could be suppressed by the loss of the other chaplins, the inactivation of the twin arginine translocation (Tat) secretion pathway, or the loss of the rodlins.

The gram-positive soil-dwelling streptomycetes have a mycelial growth habit that culminates in the formation of dormant exospores that permit survival under adverse environmental conditions (13). Germinating spores produce one or more germ tubes that grow by tip extension to form a network of branching vegetative hyphae known as the vegetative mycelium. Antibiotics (and other secondary metabolites) are produced within the vegetative hyphae, and from this vegetative mycelial network emerge specialized reproductive structures known as aerial hyphae. These aerial hyphae undergo a number of maturation steps, including a synchronous round of cell division, to differentiate into chains of unigenomic spores.

The transition from vegetative growth in an aqueous environment to the emergence of aerial hyphae into the air requires significant adaptation of the cell surface: the surfaces of vegetative hyphae are hydrophilic, while those of aerial hyphae and spores are extremely hydrophobic. Three groups of proteins are known to be involved in the modulation of cell surfaces during aerial hypha formation in *Streptomyces coelicolor*: the chaplins, the rodlins, and SapB (reviewed in references 8, 16, and 36). These proteins are thought to collectively function like the fungal hydrophobins, which are important for surface modulation and aerial growth in the filamentous fungi (reviewed in reference 37). Hydrophobins are small secreted proteins that assemble into a distinctive "rodlet" layer on the fungal cell surface; this rodlet ultrastructure is a feature shared with a number of *Streptomyces* species, including *S. coelicolor*. As their name implies, hydrophobins impart hydrophobic characteristics to the fungal cell surface that help in attachment to other surfaces (host plants, nutrient sources, etc.). They are defined by a characteristic pattern of eight cysteine residues that interact to form four intramolecular disulfide bridges but otherwise do not share significant sequence similarity with each other or with other proteins. These four disulfide bridges serve to stabilize a compact, globular protein structure with amphiphilic properties (19, 26). Hydrophobins, like the chaplins and SapB, are highly surface active and are capable of dramatically reducing surface tension at the colony air-water interface (37).

SapB was the first morphogenetic protein discovered in *S. coelicolor*, and its structure has been elucidated: it is an amphiphilic, lantibiotic-like peptide that is the product of the *ram* gene cluster (25). SapB is produced during the vegetative phase of development, and available evidence suggests that it acts as a surfactant, coating both the nascent aerial hyphae and air-water interfaces to facilitate the emergence of aerial filaments into the atmosphere (35, 36).

The chaplins are secreted proteins that, like SapB, have strong surfactant properties and have similarly been shown to coat the surfaces of aerial hyphae and spores (9, 14). The eight chaplin proteins have Sec-dependent secretion signal sequences and share a region of extensive similarity termed the chaplin domain. Five of the chaplins (the short chaplins, ChpD to -H) have a single chaplin domain, while the remaining three long chaplins (ChpA to -C) have two chaplin domains and an extended C terminus that contains a pre-

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dicted sorting signal for covalent attachment to the cell wall by a sortase enzyme. The chaplin domain itself is hydrophobic and includes two conserved Cys residues that are found in all chaplins, apart from ChpE. We have previously proposed a model for chaplin assembly in which the long chaplins act as cell wall anchors for the binding and polymerization of the short chaplins, and this polymerization ultimately results in the formation of a hydrophobic sheath encasing the aerial filaments, presumably in conjunction with SapB (5, 14). Deletion of either the SapB biosynthetic genes (the *ram* cluster) or the chaplin genes causes conditional defects in aerial hypha formation (5, 10, 28, 29), while deletion of both sets of genes results in a strain that is severely impaired in aerial development under all growth conditions (5). In addition to their role in aerial mycelium formation, the chaplins have also been implicated in the formation of the rodlet ultrastructure that decorates the surfaces of aerial hyphae and spores, together with the rodlin proteins (8, 10). Unlike the chaplins, however, the rodlins are dispensable for both aerial development and surface hydrophobicity; deletion of the rodlins simply results in a loss of the rodlet decoration on the surfaces of the aerial structures (11).

While the collective importance of the chaplins in aerial development has been firmly established, the roles played by individual chaplins are not well understood. In this work, we investigate the contributions made by the long and short chaplins to aerial hypha formation and the development of the rodlet ultrastructure, explore the importance of the conserved Cys residues, and demonstrate a unique role for ChpE in the viability of *S. coelicolor*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The *Streptomyces* strains used in this study are summarized in Table 1 and were cultured at 30°C on R5, MS, DNA, and minimal media plus glucose agar medium or in tryptone soya broth and yeast extract-malt extract liquid medium (24). *Escherichia coli* strains were typically grown at 37°C and included DH5α (for plasmid construction and routine subcloning), XL-1 Blue (for site-directed mutagenesis), ET12567/pUZ8002 (for generation of methylation-free DNA and conjugation into *Streptomyces*) (27, 31), and BW21153/pIJ790 (for PCR-targeted gene disruptions; grown at 30°C) (18). Descriptions of the plasmids used and constructed in this work are included in Table 1.

Protoplast transformation and conjugation from *E. coli* into *Streptomyces*. Introduction of DNA from *E. coli* into *S. coelicolor* requires the DNA to be passaged through the *E. coli* strain ET12567 (*dam dcm hsd*) in order to circumvent the methyl-specific restriction system of *S. coelicolor*. Protoplast generation and transformation, and plasmid conjugation, were carried out as described by Kieser et al. (24).

Construction of chaplin and rodlin mutants. *chpE* mutants and an *rdlAB* mutant were constructed as described by Elliot et al. (14, 15) according to the methods of Gust et al. (18).

Construction of "minimal chaplin" strains. The integrating plasmid vector pSET152 containing *chpC* and *chpH* was constructed as described previously (14) and was introduced into the 7× *chp* mutant (J3149A) (Table 1) by conjugation. *chpH* was obtained by digesting the plasmid pSET152+*chpCH* with EcoRI, followed by gel purification of the resulting *chpH* fragment. This DNA fragment was then ligated with pSET152, which had also been digested with EcoRI, and dephosphorylated, creating pIJ6937. This construct was also introduced into the 7× *chp* mutant (J3149A) by conjugation. *chpDA* were PCR amplified using *Pfu* DNA polymerase (Stratagene) with M600 wild-type chromosomal DNA as a template. The DNA fragment was cloned into the SmaI site of pIJ2925 before being excised with HindIII and KpnI and cloned into pMS82 digested with the same enzymes, creating pIJ6933. pIJ6933 was introduced into the 7× *chp* mutant strain alone and carrying pSET152+*chpCH* by conjugation.

TABLE 1. *S. coelicolor* strains and plasmids used in this study

Strain/plasmid	Genotype, description, or function	Reference
S. coelicolor		
M145	SCP1$^-$ SCP2$^-$	24
M600	SCP1$^-$ SCP2$^-$	6
J3149A	M600 Δ*chpAD* Δ*chpCH*::*aadA* Δ*chpB* Δ*chpF* Δ*chpG* (7× *chp*)	5
TP3	M145 Δ*tatB*::*apr*	34
TP4	M145 Δ*tatC*	34
E101	M600 Δ*chpE*::*apr tatB*::IS*1649*	This work
E102	TP4 Δ*chpE*	This work
E103	M600 Δ*rdlAB*::*vph*	This work
E104	M600 Δ*rdlAB*::*vph* Δ*chpE*::*apr*	This work
J3287	M600 Δ*ramR*::*vph*	5
J3288	M600 Δ*ramCSAB*::*vph*	5
S. lividans		
1326	SLP2$^+$ SLP3$^+$	24
10-164	1326 *msiK*	21
Plasmids		
BT340	Temperature-sensitive FLP recombination plasmid	12
pUC19	*E. coli* cloning vector	
pBluescript KS	*E. coli* cloning vector	Stratagene
pGEM-T	*E. coli* cloning vector	Promega
pIJ2925	*E. coli* cloning vector	24
pSET152	Integrative cloning vector; *ori* pUC18 *apr oriT* RK2 *int* ϕC31 *attP* ϕC31	4
pIJ82	pSET152 derivative (hygromycin resistant)	
pMS82	Integrative cloning vector; *hyg oriT int* ϕBT1 *attP* ϕBT1	17
pIJ6916	pIJ2925+p*vanJ*+*chpE*	This work
pIJ6917	pIJ82+p*vanJ*+*chpE*	This work
pIJ6937	pSET152+*chpH*	This work
pIJ6936	pSET152+*chpH**	This work
pIJ6935	pSET152+*chpCH*	14
pIJ6934	pSET152+*chpCH**	This work
pIJ6933	pMS82+*chpDA*	This work
pMC101	pMS82+*chpABC*	This work
pTDW47	pSET152+*dagA*	34
pTDW46	pSET152+*dagA* (signal peptide sequence replaced with *aadA*)	34

Site-directed mutagenesis of *chpH*. *chpH* was excised as an EcoRI fragment from pBluescript containing *chpCH* and was cloned into pUC19 digested with EcoRI. The resulting plasmid (pUC+*chpH*) was used as a template for the PCR-based mutagenesis of the two Cys residues in *chpH* (C56 and C74). The complementary primers Cys56Val 1 and Cys56Val 2 (Table 2) were used to amplify the entire plasmid using *Pfu* enzyme (Stratagene), changing the Cys (TGC) at amino acid position 56 to a valine (GTC). The PCR program was 95°C for 5 min and then 5 cycles of 95°C for 1 min, 65°C for 1 min, and 72°C for 10 min; 19 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 10 min; followed by a final elongation at 72°C for 15 min. Next, the template DNA was selectively cleaved by digestion with DpnI (which recognizes and cleaves methylated DNA) for 2 h at 37°C. Three microliters of the reaction mixture was then used for electroporation into *E. coli* XL-1 Blue, and positive transformants were selected by plating them on LB containing 100 μg/ml ampicillin. Four colonies were selected for further examination through plasmid isolation and sequencing. One of the four (pUC+H C56V) had the correct mutation introduced, with no additional sequence changes, so it was subjected to a second round of mutagenesis. The complementary primers Cys74Gly 1 and Cys74Gly 2 were used to amplify the entire pUC+H C56V plasmid, as outlined above, to change the Cys (TGC) at amino acid position 74 to a glycine (GGC) residue. Again, sequencing was conducted to ensure that only the desired mutation was introduced. The mutagenized *chpH* gene

TABLE 2. Oligonucleotide primers used in this study

Name	Sequence (5′ to 3′)[a]	Function
Cys56Val 1	GGTGAACGTCGTCGGCAACACG	Mutagenesis of *chpH*
Cys56Val 2	CGTGTTGCCGACGACGTTCACC	Mutagenesis of *chpH*
Cys74Gly 1	CCTTCGGCAACGTCGGCATCAACAAGTGACG	Mutagenesis of *chpH*
Cys74Gly 2	CGTCACTTGTTGATGCCGACGTTGCCGAAGG	Mutagenesis of *chpH*
chpC end	CGAGTACGGACACTGGGAG	Cloning of *chpCH*
chpH up	CGGAGTGGACGAGCGGGTGC	Cloning of *chpCH*
chpD up	GCTGTCGGCGAACGGCGAGG	Cloning of *chpAD*
chpA end	CGCCTCTAGACCCTGCACCTGGACCTGACC	Cloning of *chpAD*
*tatB*1	CCCTCGACATGAACTACACGG	PCR amplification of *tatB*
*tatB*2	GCGTCACGTCCTGGATGACC	PCR amplification of *tatB*
*tatB*3	GCCTCCTCGCTCTCGTCGG	PCR amplification of *tatB*
chpE up	GGGCAGATCTGCGACTGCCGCGGCGATCG	Cloning of *chpE*
chpE end	GGAGCGGGGGCGGTGACCG	Cloning of *chpE*
chpE Fwd	GGCGACTGCCGCGGCGATCGCAAGGAGGGGTTGTAAGTG ATTCCGGGGATCCGTCGACC	Knockout of *chpE*
chpE Rev	GCCCGCTGCTGAGGCGGCATTGGGGGGCGGCCTGCGTCA TGTAGGCTGGAGCTGCTTC	Knockout of *chpE*
rdlAB Fwd	AAGTCAGCGGGCCGCCCGTACCGGGCTGGGCTGGGCTCA ATTCCGGGGATCCGTCGACC	Knockout of *rdlAB*
rdlAB Rev	CCGCCGAAGTGCTCGGCGGCCCGCCCCGGGGCGATGTCA TGTAGGCTGGAGCTGCTTC	Knockout of *rdlAB*
rdlA NdeI	GGCGCGCATATGCTCAAGAAGGCAATGGTC	Cloning of RdlA signal peptide
rdlA BamHI	GGCGCGGGATCCCACGGCCGGCCCGTTGTC	Cloning of RdlA signal peptide
rdlB NdeI	GGCGCGCATATGAGCTCCGGTGGACGGGGTTTC	Cloning of RdlB signal peptide
rdlB BamHI	GGCGCGGGATCCGCTGTCGTCGCCGATCGC	Cloning of RdlB signal peptide

[a] Engineered restriction endonuclease recognition sequences are underlined.

(*chpH**) was then excised as an EcoRI fragment and was introduced into either pSET152 (creating pIJ6936) or pSET152+*chpC* cut with EcoRI (creating pIJ6934) before being conjugated into *S. coelicolor* J3149A.

Construction of *chpE* complementation constructs. The vancomycin-inducible promoter of *vanJ* (*vanJp*) was excised from pIJ6882 (20) with HindIII and SalI and was cloned into pIJ2925 digested with the same enzymes. *chpE* was PCR amplified using primers *chpE* up and *chpE* end (Table 2) and was cloned into the vector pGEM-T (Promega). *chpE* was removed using BglII and SacI and was cloned into pIJ2925+p*vanJ* digested with BamHI and SacI, creating pIJ6916. The *vanJp-chpE* fragment was excised from pIJ2925 using BglII and was introduced into pIJ82 (a kind gift from H. Kieser), which is a derivative of pSET152 in which the apramycin resistance gene has been replaced by a hygromycin resistance gene. This plasmid (pIJ6917) was then introduced into *S. coelicolor* M600. The *chpE* knockout cosmid was then introduced into the plasmid-containing strain, and the resulting colonies were screened for the creation of a *chpE* null mutant in the presence (10 μg/ml) or absence of the inducer, vancomycin.

Agarase assay. The agarase assay for detection of Tat-dependent signal peptides was conducted as described by Widdick et al. (34). Sequences corresponding to the RdlA and RdlB signal peptides were introduced upstream of the leaderless *dagA* gene in pTDW46. These constructs were introduced into *Streptomyces lividans* 10-164 by conjugation. pTDW47, containing an intact *dagA* gene (with its associated leader peptide), was also introduced into *S. lividans* 10-164 as a positive control for agarase activity. Agarase production rates in *S. lividans* 10-164 alone and containing pTDW46-RdlA, pTDW46-RdlB, and pTDW47 were compared. Ten thousand spores of each strain were spotted on minimal medium plus glucose agar medium plates and were allowed to grow for 72 h before the plates were stained with Lugol solution (VWR) for 45 min. Zones of clearing indicated agarase activity.

Scanning electron microscopy. For scanning electron microscopy, colonies were mounted on the surface of an aluminum stub with optimal cutting temperature compound (Miles Scientific), plunged into liquid nitrogen slush at approximately −210°C to cryopreserve the material, and transferred to the cryostage of an Alto 2500 cryotransfer system (Gatan, Oxford, England) attached to a Zeiss Supra 55 VP field emission gun scanning electron microscope (Zeiss SMT, Germany). The surface frost was sublimated at −95°C for 3 min before the sample was sputter coated with platinum for 2 min at 10 mA at below −110°C. Finally, the sample was moved onto the cryostage in the main chamber of the microscope, held at approximately −130°C, and viewed at 1.2 to 5.0 kV. Scanning electron microscope images were saved as TIF graphic files and manipulated in Adobe Photoshop 7.0.

RESULTS

Creation of a "minimal chaplin strain." Deletion of individual chaplin genes (*chpH* or *chpB*) or pairs of genes (*chpCH* or *chpAD*) had no obvious phenotypic effect on colony growth or development (14), suggesting significant redundancy in chaplin function. To determine the extent of chaplin redundancy, we attempted to develop a "minimal chaplin strain" that would raise an abundant aerial mycelium by reintroducing individual chaplin genes into a *chp* mutant strain containing only *chpE* (the 7× *chp* mutant J3149A). *chpC*, *chpE*, and *chpH* are the only chaplin genes conserved in all *Streptomyces* species whose genome sequences are available (2, 22, 29a) (www.sanger.ac.uk/Projects/S_scabies/). In addition, previous transcript analyses showed that *chpC*, *chpE*, and *chpH* were the only chaplins expressed prior to the initiation of aerial hypha formation, with *chpE* and *chpH* expressed at continuously high levels throughout the developmental cycle (9, 14). As such, it seemed likely that these three chaplins would make a major contribution to aerial hypha development. A strain containing only *chpE* (Fig. 1A) had a bald colony phenotype identical to that of the complete chaplin (8× *chp*) mutant strain (5), as did a strain containing only the genes for the long chaplins (*chpA*, *chpB*, and *chpC*) (data not shown). Introduction of *chpH* into the *chpE*-containing mutant background, however, resulted in a strain that could raise a sparse aerial mycelium on MS medium after extended incubation (Fig. 1B). In contrast, when we introduced *chpC* and *chpH* together into the *chpE*-containing 7× mutant, the resulting strain developed a robust, sporulating aerial mycelium (Fig. 1C),

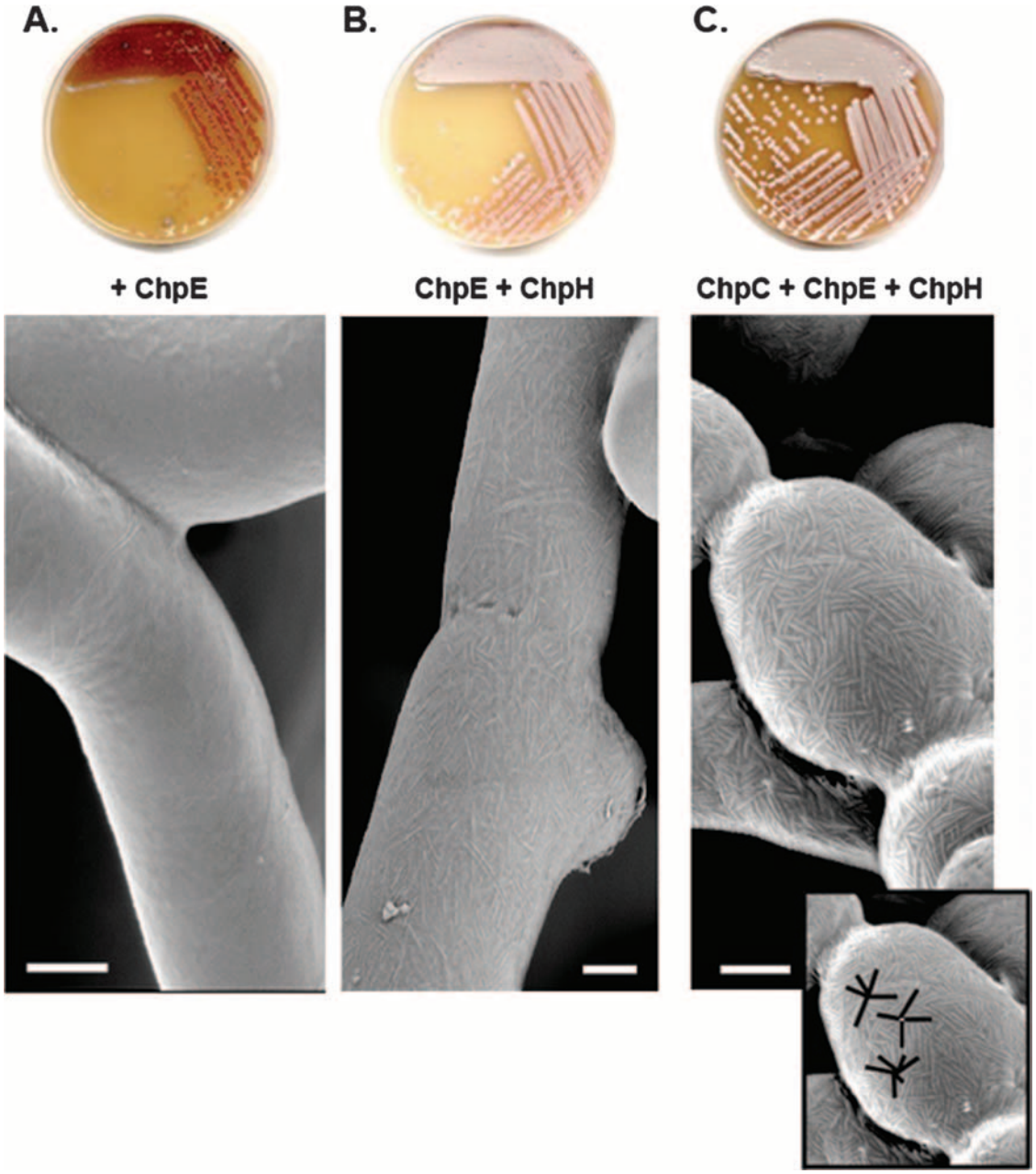

FIG. 1. The introduction of additional chaplin genes into the 7× *chp* mutant strain (J3149A), which contains only *chpE*, results in increased aerial hypha formation (plate images) and increased surface ultrastructure (scanning electron micrographs). (A) J3149A. (B) J3149A with *chpH* introduced. (C) J3149A with *chpC* and *chpH* introduced. (Inset) Starburst rodlet pattern on the spore surface. Images were taken after 7 days of incubation. Bars = 250 nm.

albeit with growth kinetics similar to those of the *chpE*-and *chpH*-containing strain (i.e., at a lower rate than the wild-type strain). Thus, expression of the long chaplin ChpC, in combination with the short chaplins ChpE and ChpH, was sufficient to promote the growth of an abundant aerial mycelium, demonstrating redundancy among the chaplins but suggesting that both long and short chaplins play important roles in aerial development. To determine whether the presence of any long chaplin and short Cys-containing chaplin was sufficient to promote aerial hypha formation, we introduced *chpA* (encoding a long chaplin) and *chpD* (encoding a Cys-containing short chaplin) into the strain containing only *chpE*. We observed significant aerial hypha formation, although not as robust as with *chpC* and *chpH* (data not shown), likely due to the fact that *chpA* and *chpD* are not as highly expressed at all times as *chpC* and *chpH* (14).

As the loss of all eight chaplin genes eliminated rodlet formation (5, 10), we examined the surfaces of the aerial hyphae and spores of strains containing *chpEH* and *chpCEH* to see if they had regained the characteristic rodlet ultrastructure. We found that *chpE* and *chpH* together restored the formation of

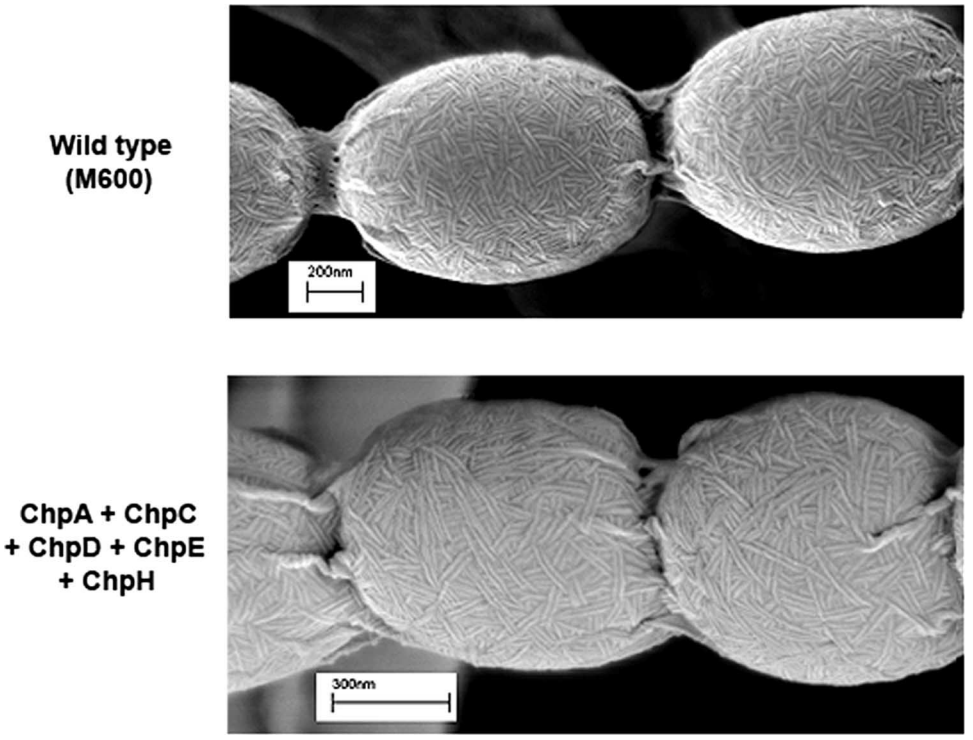

FIG. 2. Scanning electron micrographs comparing the surface ultrastructure of wild-type *S. coelicolor* M600 (top) with that of a 7× *chp* mutant strain (containing only *chpE*) to which *chpAD* and *chpCH* were introduced on integrating plasmid vectors (bottom).

sparse but well-defined individual surface fibers (Fig. 1B), although we did not observe any of the paired rodlet filaments typical of the wild type. The addition of *chpC* significantly enhanced the formation of both individual and paired surface fibers, and these were frequently arranged in a "starburst-like" pattern, with multiple fibers appearing to emanate from a central point (Fig. 1C). This provides direct experimental evidence that the chaplins are major contributors to the rodlet ultrastructure on aerial surfaces. Furthermore, we found that the introduction of either two copies of *chpCH* (one copy on each of two independently integrating plasmid vectors) (data not shown) or *chpCH* together with *chpAD* (Fig. 2) into the *chpE*-containing J3149A strain resulted in near-wild-type abundance of paired rodlets, in contrast to the minimal chaplin strain; however, these rodlets did not show the same degree of organization as those of the wild type.

The formation of aerial hyphae and surface fibers depends upon conserved Cys residues in ChpH. Given that the *chpCEH*-containing strain could raise an abundant aerial mycelium and assemble a rodlet ultrastructure on aerial surfaces, we decided to use the strain to probe the role of the short chaplins and, in particular, the Cys-containing short chaplins. The chaplin domains of all short chaplins, apart from ChpE, contain two highly conserved Cys residues (Fig. 3). Previous work suggested that these Cys residues formed intramolecular disulfide bonds in the mature extra-

FIG. 3. Alignment of the short-chaplin domains from *S. coelicolor* (sc), *S. avermitilis* (av), *S. griseus* (sg), and *S. scabies* (ss). The sequences shown represent the processed (signal peptide removed), mature chaplin form. Identical amino acid residues are highlighted in black, and similar amino acid residues are shown in gray. The arrows indicate the sites of conserved Cys residues.

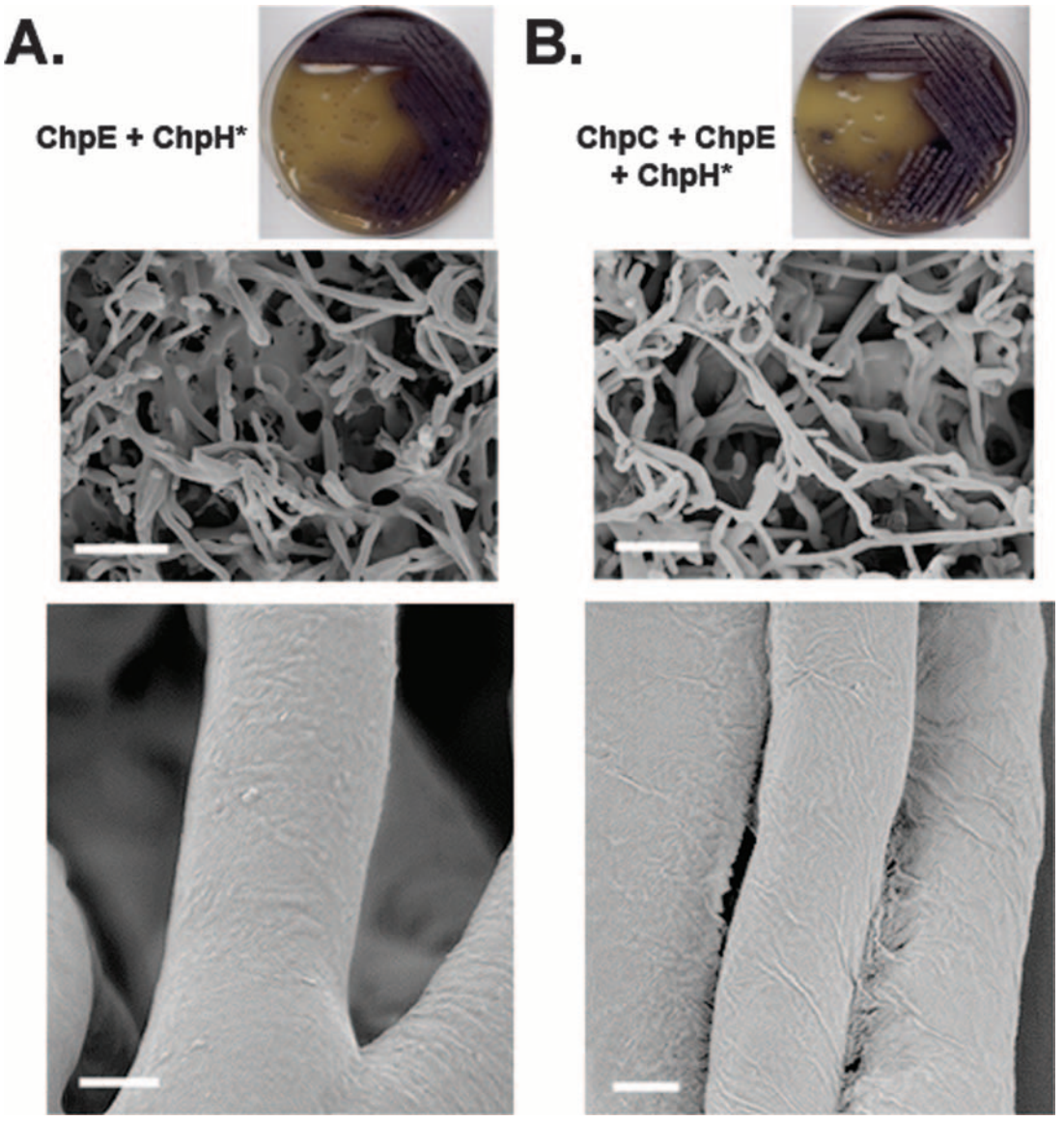

FIG. 4. The phenotypic effect of removing the two Cys residues in ChpH. (A) A strain producing only ChpE and ChpH* is largely incapable of raising aerial hyphae (top and middle) and exhibits no rodlet ultrastructure (bottom). (B) A strain producing ChpC, ChpE, and ChpH* is capable of raising slightly more hyphae than the strain shown in panel A but is still largely defective in aerial hypha formation (top and middle) and is also devoid of rodlet fibers on the aerial surfaces (bottom). Bars: middle, 5 μm; bottom, 200 nm.

cellular chaplin proteins (14), and it is well established that analogous disulfide bonds in the fungal hydrophobins stabilize their amphiphilic structure and are critical for function (19, 26). To investigate the importance of these residues in chaplin function, we replaced the two Cys residues in ChpH with a glycine and a valine (these residues are found in place of the Cys residues in ChpE in *S. coelicolor* and thus were deemed unlikely to disrupt chaplin stability) (Fig. 3). The Cys-free ChpH-encoding gene (*chpH**) was then introduced into the *chpE*-containing mutant strain either alone or in conjunction with *chpC*. The two resulting strains formed a very sparse aerial mycelium (Fig. 4) compared with the otherwise identical strains carrying the wild-type *chpH* gene (Fig. 1) (see above). Examination of the aerial surfaces of these two mutant strains also revealed a complete absence of rodlet ultrastructure (Fig. 4). This suggested that the Cys residues in ChpH were necessary both for aerial hypha formation and for assembly of the rodlet ultrastructure. To exclude the possibility that ChpH* had a dominant-negative effect on aerial hypha formation, we compared the effects of introducing *chpH** versus *chpH* into a wild-type strain. No phenotypic differences were observed (data not shown), suggesting that ChpH* does not inhibit aerial morphogenesis.

***chpE* is essential.** ChpE differs from the other short chaplins in that it lacks the two highly conserved Cys residues important for ChpH function (Fig. 3). In addition, ChpE has a different hydrophobicity profile, with alanine replacing valine or leucine at multiple positions (e.g., at residues 18,

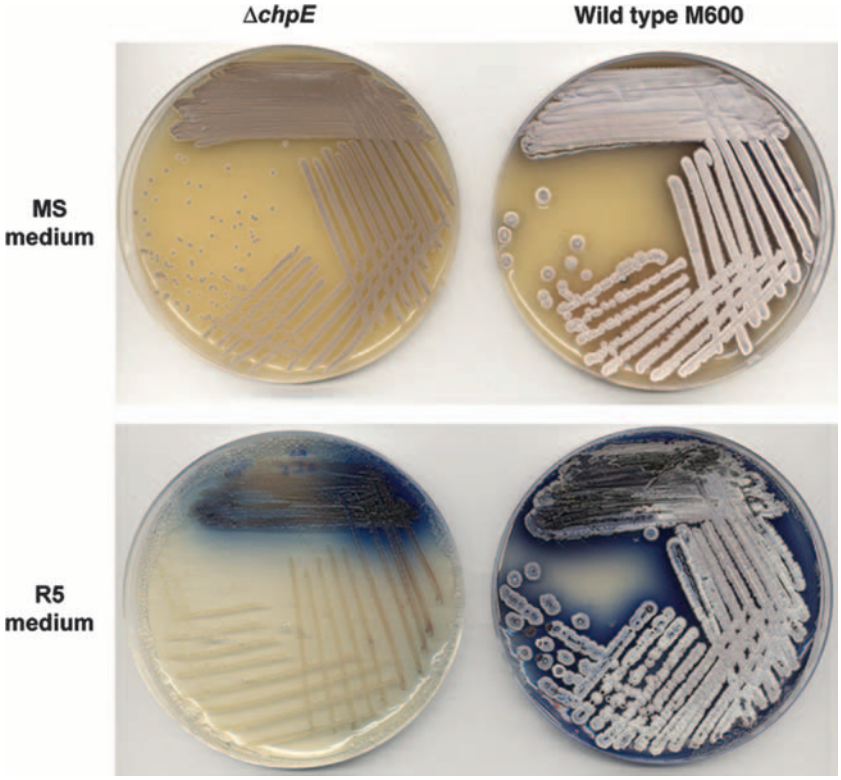

FIG. 5. Phenotypic comparison of wild-type *S. coelicolor* M600 with the constructed *chpE* null mutant (carrying an insertion element in *tatB*). The top panel shows the two strains grown on MS medium, while the bottom panel shows the two strains grown on rich R5 medium.

25, and 33 in the mature *S. coelicolor* ChpE). These characteristics are conserved in the ChpE orthologues from *Streptomyces avermitilis* (SAV6478), *Streptomyces griseus* (SGR5696) and *Streptomyces scabies* (Fig. 3), suggesting that ChpE has a unique function among the chaplins. Our creation of the minimal chaplin strain did not reveal a function for ChpE, as a strain containing only ChpE looked identical to one lacking all the chaplins. Furthermore, ChpE could not substitute for ChpH* in stimulating aerial hypha formation. Therefore, we decided to investigate ChpE function in a wild-type background. We tried to create a *chpE* mutant in the wild-type M600 strain by replacing the chromosomal *chpE* gene with an apramycin resistance cassette, using the robust Redirect PCR-targeting method of Gust et al. (18), but were repeatedly unable to do so. We and others had successfully deleted *chpE* in strains already lacking other *chp* genes (5, 9), which suggested either a technical problem or that *chpE* was essential in a wild-type genetic background. To distinguish between these two possibilities, we introduced a second copy of *chpE*, under the control of a vancomycin-inducible promoter, into the wild-type *S. coelicolor* strain M600. In the presence of vancomycin, we were readily able to disrupt the native chromosomal copy of *chpE* (108/736 colonies screened), whereas in the absence of vancomycin, no double-crossover gene replacements were obtained (0/522 colonies screened), providing strong evidence that *chpE* is essential in a wild-type genetic background. Attempts to grow the knockout strains on solid medium in the absence of vancomycin induction resulted in the frequent appearance of colonies that had acquired compensatory suppressor mutations. These colonies were heterogeneous in their appearance: many had a wild-type appearance, while others had an unusual small-colony phenotype (see below).

Suppressors of *chpE* mutation. The above observations suggested that *chpE* was essential in a wild-type background; however, *chpE* was successfully deleted from a strain that was missing all of the other chaplin genes (5, 10) and also from a strain that was missing all of the chaplin genes apart from *chpF* and *chpG* (9). This suggested that the essential nature of *chpE* might be conditionally dependent upon the presence of particular *chp* genes. One model that could account for these observations is that ChpE is involved in coordinating the assembly or polymerization of the other chaplins. To test this hypothesis, we focused on *chpC* and *chpH*, two of the most highly expressed and most conserved (apart from *chpE* itself) of the chaplin genes, and tested whether it was possible to introduce *chpC* and *chpH* together into an 8× *chp* mutant strain lacking *chpE*. We found that we were able to readily construct this strain, implying that the essential nature of *chpE* is not tied to the expression and function of *chpC* and *chpH*. We also tested the relative ease with which we could create a *chpE* mutant in a variety of other developmental mutant strains, including Δ*ramR* and Δ*ramCSAB* strains, which are both unable to produce SapB, and a Δ*rdlAB* strain, which is unable to produce the two rodlin proteins, RdlA and RdlB. We were unable to create a *chpE* mutant in either of the *ram*

mutant strains, but we could easily knock out *chpE* in the rodlin mutant background (8/25 colonies screened).

Second-site suppressors of *chpE* are found in the Tat secretion system. During the course of our attempts to create a *chpE* knockout in the wild-type genetic background, we identified several colonies that had the correct antibiotic resistance profile for a *chpE* knockout and confirmed that these colonies represented *chpE* null mutations using PCR and Southern blot analyses (data not shown). Interestingly, however, these mutants had a phenotype distinct from those of wild-type strains (Fig. 5) and characterized *rdl* and *chp* mutant strains (5, 9, 10, 11). On MS medium, the mutants had a small-colony phenotype and did not produce the secreted hydrolytic enzyme agarase (which, in the wild-type strain, gives rise to craters in the agar surrounding the colonies); on rich (R5) medium, the colonies were "bald" (devoid of aerial hyphae) and produced reduced levels of the blue-pigmented antibiotic actinorhodin; in high-sucrose liquid culture (yeast extract-malt extract) the mutants failed to grow; and in rich liquid medium (tryptone soya broth), the mutants grew in a very dispersed manner, more reminiscent of *E. coli*-type growth than the pellet-like mycelial growth typical of *Streptomyces*. None of these mutant phenotypic characteristics could be complemented by the introduction of a wild-type copy of *chpE*, suggesting that the unusual phenotypes were due to a second-site suppressor mutation rather than the *chpE* null mutation itself.

Intriguingly, the mutant phenotype was very similar to that of strains carrying mutations in the twin arginine (Tat) secretion pathway (32, 34), with agarase being a known Tat substrate (34). Unlike the Sec secretion pathway, which translocates proteins in an unfolded conformation, the Tat pathway is dedicated to the translocation of folded substrates, many of which contain cofactors. Secretion through the Tat pathway requires three membrane-localized proteins: TatA, TatB, and TatC. The mutation of genes encoding any of these three proteins confers a phenotype seemingly identical to that of the *chpE* suppressor mutants.

To determine whether the *chpE* mutant had defects in any of the Tat translocation components, we PCR amplified *tatA*, *tatB*, and *tatC* from the *chpE* suppressor mutants and sequenced each of the resulting products. For several independent mutant isolates, there was an insertion element (IS*1649*) disrupting the coding sequence of *tatB*. This suggested that inactivation of the Tat pathway suppressed the lethality of the *chpE* null mutation and, additionally, that there may be an insertion "hot spot" within the 5′ end of *tatB*, given the isolation of several independent suppressor strains carrying IS*1649* inserted at an identical location, 26 nucleotides downstream from the start of the coding sequence.

Loss of a functional Tat secretion system permits deletion of *chpE*. To further examine the connection between the Tat secretion system and *chpE*, we attempted to create a *chpE* gene knockout in a Tat mutant background, using a *tatC* null mutant (34). We found that *chpE* null mutants could be readily constructed in this mutant background (6/32 colonies screened). This confirmed that inactivation of the Tat pathway suppressed the lethality of the *chpE* null mutation and, furthermore, showed that the connection to *chpE* was not specific to *tatB*. There is no obvious direct link between the chaplins and Tat secretion, given that all the chaplins have typical Sec-dependent signal sequences. However, it was formally possible that a *tat* mutation might indirectly prevent secretion of the other chaplin proteins, thereby suppressing the lethality of *chpE* disruption. To address this possibility, we isolated cell wall fractions of sporulating cultures of a *tatB chpE* double mutant and followed the chaplin purification procedure used previously for the isolation and identification of the short chaplins (14). Using matrix-assisted laser desorption ionization–time of flight mass spectrometry, we successfully identified all of the four remaining short chaplins in these cell surface fractions, suggesting that chaplin secretion is not impaired in a *tatB chpE* double mutant (Fig. 6).

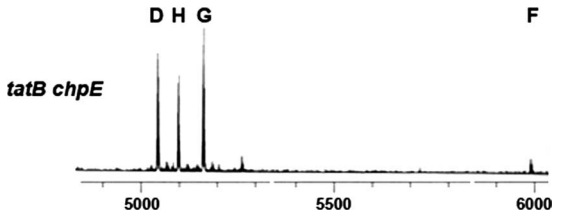

FIG. 6. Matrix-assisted laser desorption ionization–time of flight mass spectrometry of cell wall extracts isolated from the *tatB chpE* double mutant grown on MS medium. The peaks corresponding to ChpD, ChpH, ChpG, and ChpF are labeled. The *x* axis represents the mass (*m*)/charge (*z*) ratio, where $z = 1$.

RdlA and RdlB are not Tat substrates. As it was straightforward to create a *chpE* knockout in a *rdlAB* mutant background, we examined the sequences of the signal peptides of RdlA and RdlB to determine whether either was a potential Tat substrate, thus providing a potential link between the Tat system and *chpE*. The RdlB leader sequence revealed a potential Tat-dependent signal peptide, as predicted by the TatP 1.0 server (1), although it had a degenerate "Tat motif" at its extreme N terminus (G-R-X-F-L as opposed to the prototypical R-R-X-[FGAVML]-[LITMVF]). The same program did not predict a Tat-dependent signal peptide for RdlA. To test whether RdlB or RdlA was a Tat substrate, we used an agarase assay that had been developed to differentiate proteins secreted via the Sec secretion system from those secreted via the Tat secretion system (34). Agarase, encoded by the *dagA* gene, is functional only when secreted by the Tat system in *S. coelicolor*. The agarase assay involves replacing the native DagA signal peptide with a signal peptide of interest (in this case, the RdlA and RdlB signal peptides) and monitoring the secretion of functional agarase upon introduction into a non-agarase-producing strain (*S. lividans* 10-164). The production and secretion of agarase are monitored using Lugol solution, an iodine-based dye that stains agar a dark-brown color, apart from areas of agarase activity, which are detected as zones of clearing around producing colonies. We introduced the RdlA-DagA and RdlB-DagA fusion constructs, along with DagA carrying its native signal peptide as a positive control, into *S. lividans* 10-164. We found that there was no agarase activity detected when it was fused to the RdlA or RdlB signal peptides (Fig. 7A). This suggested that neither rodlin signal peptide was capable of driving agarase secretion through the Tat system and that RdlA and RdlB were instead likely substrates for the Sec secretion system.

To further investigate whether RdlA or RdlB was a Tat substrate, we examined the surface of a *tatB* mutant using

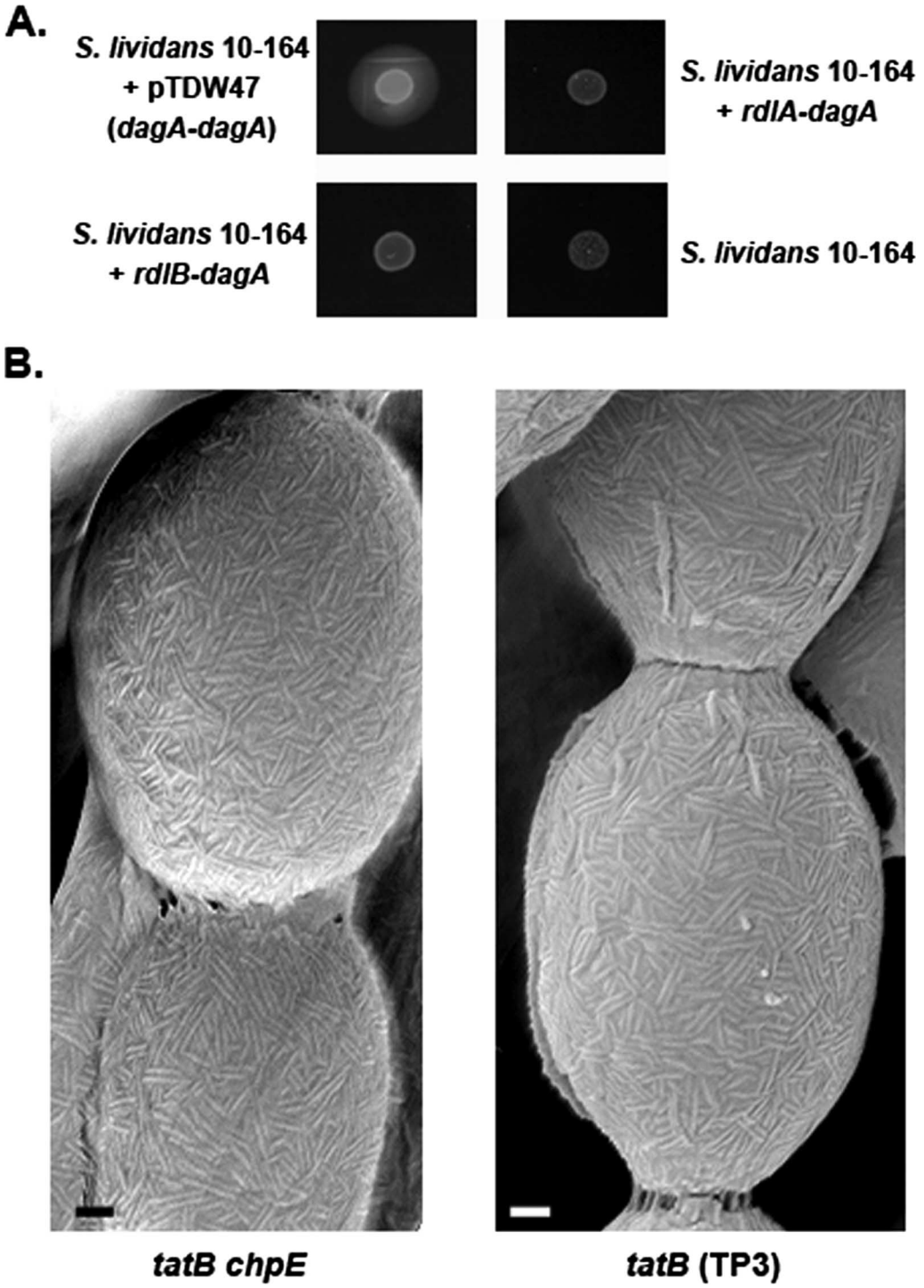

FIG. 7. (A) Agarase assay in *S. lividans* 10-164 comparing secretion of agarase with its native signal peptide (top left), RdlA signal peptide (top right), and RdlB signal peptide (bottom left). *S. lividans* 10-164 alone served as a negative control (bottom right). (B) Scanning electron micrographs showing the surfaces of the *tatB chpE* double mutant and the *tatB* (TP3) mutant. Abundant rodlet ultrastructure is evident for both strains. Bars = 100 nm.

high-resolution scanning electron microscopy. RdlA and RdlB are not functionally redundant, as both proteins are required for the assembly of a rodlet ultrastructure on aerial surfaces (10). We found that both a *tatB* mutant strain and a *tatB chpE* double-mutant strain had significant rodlet ultrastructure on their surfaces, albeit appearing slightly less organized than that of a wild-type strain (Fig. 7B). This implied that the Tat secretion system plays a novel, as yet uncharacterized role in chaplin fiber assembly, independent of chaplin export to the surface and interaction with the rodlins.

DISCUSSION

Model for the activity of long and short chaplins in aerial hypha and rodlet formation. In our original model of chaplin activity (14), we proposed that the long chaplins, believed to be covalently attached to the cell wall by a sortase enzyme(s), serve as primary (but not exclusive) anchors for the short chaplins on the cell surface through the heteropolymerization of chaplin domains. This was predicted to result in the formation of a hydrophobic surface layer that would help to break surface tension during the erection of aerial hyphae and would also help to prevent desiccation of the aerial filaments. In addition to modulating surface hydrophobicity, work by Claessen et al. (10) has suggested that the chaplins also interact with the rodlin proteins to generate the characteristic rodlet pattern found on the surfaces of aerial structures. We used our minimal chaplin strain to examine the roles played by the long and the short chaplins and to test our model of chaplin activity.

In the minimal chaplin strain, the short chaplin ChpH appears to be the main polymerization unit driving aerial hypha and rodlet formation. Neither ChpE nor ChpC contributes significantly to either of these processes in the absence of a functional ChpH; however, ChpC greatly enhances both the formation of aerial hyphae and the assembly of a rodlet ultrastructure when introduced in conjunction with ChpH and ChpE. This would be consistent with a role for ChpC as an anchor, rather than a significant polymerization unit—a role that is further supported by our observation that a strain containing only the long chaplins has a phenotype very similar to that of an 8× *chp* mutant. Examination of the aerial structures of strains containing ChpC, ChpE, and ChpH revealed increased rodlet fibrils and a greater organization of these fibrils than was found in the absence of ChpC. A striking feature of these ChpC-containing strains is the appearance of foci from which many fibers can be seen to emanate. This, too, is consistent with an anchoring role for ChpC in the polymerization of the short chaplins. We suggest that these functions of ChpC as a cell wall anchor and ChpH as a polymerization unit are likely to be representative of the functions of the other long chaplins and the other Cys-containing short chaplins, respectively. In support of this, a strain expressing ChpADE (where ChpA is a long chaplin and ChpD is a Cys-containing short chaplin) could also raise aerial hyphae and assemble a rodlet ultrastructure (data not shown).

Cysteines and disulfide bond formation. An important difference between ChpE and the remaining short chaplins is the absence of two Cys residues in ChpE that are conserved in the other four. These Cys residues are integral to the function of ChpH, as their removal resulted in greatly reduced aerial hypha formation and a complete abrogation of rodlet assembly in the minimal chaplin strain. Our previous work suggested that these Cys residues form intramolecular disulfide bonds in the mature extracellular ChpH and in the other Cys-containing short chaplins (14). We propose that these disulfide bonds play a critical role in the stabilization of chaplin structure, perhaps by locking the proteins in an amphipathic conformation; the amino acids between the two Cys residues are predominantly hydrophobic and could form a hydrophobic patch having the capacity to confer amphipathic properties on the chaplin domain.

Hydrophobins, which are involved in rodlet assembly and aerial hypha formation in the filamentous fungi, have eight highly conserved Cys residues that form four intramolecular disulfide bonds. Loss of the eight Cys residues from the MPG1 hydrophobin of *Magnaporthe grisea* results in defects in aerial hypha formation (23) that are similar to those we observed after loss of the Cys residues from ChpH. In contrast to the ChpH*-containing strain, however, loss of the Cys residues in the fungal hydrophobins did not disrupt rodlet assembly, suggesting that, unlike for the chaplins, disulfide bond formation is not a prerequisite for fungal hydrophobin self-assembly. The disulfide bonds formed in the fungal hydrophobins are known to be important structural determinants: they reduce conformational flexibility and stabilize the globular, amphiphilic structure of the proteins (19, 26). A similar compact, amphiphilic structure is adopted by the *Streptomyces* morphogenetic surfactant peptide SapB. Instead of disulfide bonds, however, SapB forms lanthionine bridges, which impose flexibility constraints and result in the exposure of surface-localized hydrophobic side chains, thus forming an amphipathic molecule (25).

ChpE serves a unique function among the chaplins. The inability of ChpE to form intramolecular disulfide bonds, due to the absence of the conserved Cys residues, suggested that it would have a unique structure relative to the other chaplins. The fact that *chpE* is essential in a wild-type genetic background implies that ChpE also has a unique function. As *chpE* is expressed early in development, it is possible that ChpE has a vital role, independent of the other chaplins, in early colony development. However, ChpE is dispensable in *tat* mutants, an *rdlAB* mutant, and strains lacking the other chaplin genes, suggesting that vegetative growth is not dependent upon ChpE function. Instead, we propose that ChpE coordinates the assembly and/or polymerization of the other chaplins, possibly by mediating their interaction with the rodlin proteins, and that loss of this coordination is lethal to the developing *Streptomyces* colony. The abundance of other chaplin proteins appears to be key, as *chpE* disruption can be accomplished in a variety of chaplin mutant backgrounds, as shown here and in previous work (5, 9). It is interesting that, although the number of chaplin genes present in *Streptomyces* species is variable, ChpE is conserved in all sequenced *Streptomyces* genomes available thus far.

The apparent functional difference, but extensive sequence similarity, between ChpE and the other short chaplins is reminiscent of the curli system in *E. coli*. Curli fibers, like chaplin fibers, are amyloid-like structures (7, 33) that are formed through the polymerization of two homologous proteins: CsgA and CsgB. These two proteins share significant sequence similarity; however, CsgA has been identified as the main curli polymerization unit (30), while CsgB functions primarily as the nucleator for the polymerization of CsgA (3). Whether such functional differentiation exists between ChpE and ChpH remains to be seen.

The connection between ChpE and the Tat secretion system is intriguing but obscure. Neither the chaplins nor the rodlins are Tat substrates, and their secretion is not affected in a *tatB* mutant, yet mutations in the *tat* genes suppress the lethality of

the *chpE* null mutation in an otherwise wild-type genetic background. The most likely explanation for these observations is that a Tat-dependent substrate(s) contributes to ChpE-dependent colony viability; however, the nature of this connection awaits further investigation.

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