SapB and the chaplins: connections between morphogenetic proteins in *Streptomyces coelicolor*

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Summary

Morphogenesis in the streptomycetes features the differentiation of substrate-associated vegetative hyphae into upwardly growing aerial filaments. This transition requires the activity of *bld* genes and the secretion of biosurfactants that reduce the surface tension at the colony–air interface enabling the emergence of nascent aerial hyphae. *Streptomyces coelicolor* produces two classes of surface-active molecules, SapB and the chaplins. While both molecules are important for aerial development, nothing is known about the functional redundancy or interaction of these surfactants apart from the observation that aerial hyphae formation can proceed via one of two pathways: a SapB-dependent pathway when cells are grown on rich medium and a SapB-independent pathway on poorly utilized carbon sources such as mannitol. We used mutant analysis to show that while the chaplins are important, but not required, for development on rich medium, they are essential for differentiation on MS (soy flour mannitol) medium, and the corresponding developmental defects could be suppressed by the presence of SapB. Furthermore, the chaplins are produced by conditional *bld* mutants during aerial hyphae formation when grown on the permissive medium, MS, suggesting that the previously uncharacterized SapB-independent pathway is chaplin dependent. In contrast, a *bld* mutant blocked in aerial morphogenesis on all media makes neither SapB nor chaplins. Finally, we show that a constructed null mutant that lacks all chaplin and SapB biosynthetic genes fails to differentiate in any growth condition. We propose that the biosurfactant activi-

ties of both SapB and the chaplins are essential for normal aerial hyphae formation on rich medium, while chaplin biosynthesis and secretion alone drives aerial morphogenesis on MS medium.

Introduction

The developmental life cycle of the sporulating, filamentous, Gram-positive soil bacterium *Streptomyces coelicolor* is a valuable model for the study of prokaryotic differentiation (as reviewed in Chater and Horinouchi, 2003). Growth of *S. coelicolor* begins with spore germination and hyphal outgrowth, which leads to the establishment of a vegetative, or substrate, mycelium. The sensing of nutrient deprivation is thought to stimulate reproductive growth, which results in the emergence of aerial hyphae known collectively as an aerial mycelium, in which chains of spores develop. Much of the research into *S. coelicolor* development has focused on the complex regulatory networks that govern the differentiation of vegetative hyphae into upwardly growing aerial hyphae (Kelemen and Buttner, 1998; Chater and Horinouchi, 2003). In contrast, little was known about the actual mechanics of aerial hyphae formation until very recently (Elliot and Talbot, 2004; Claessen *et al.*, 2006; Willey *et al.*, 2006). Importantly, it appears that the surface tension between the aqueous milieu of the colony and the air must be reduced if nascent hyphae are to grow into the air, and this can be accomplished by two classes of surfactant-like molecules: the SapB peptide (Tillotson *et al.*, 1998; Kodani *et al.*, 2004) and the chaplin proteins (Claessen *et al.*, 2003; Elliot *et al.*, 2003a).

SapB is an amphiphilic, 'lantibiotic-like' lanthionine-containing peptide (Kodani *et al.*, 2004). Lantibiotics are ribosomally synthesized oligopeptide antibiotics produced by Gram-positive bacteria that are translated as inactive prepeptides, and undergo extensive post-translational modification before being cleaved to yield the mature peptide (Sahl and Bierbaum, 1998). Unlike most lantibiotics, SapB does not appear to have antibiotic activity. Instead, it is secreted onto the colony surface where it is thought to function as a biosurfactant, facilitating the upward growth of emerging aerial hyphae. A similar lantibiotic-like structure and role have been ascribed to SapT, produced by *Streptomyces tendae* (Kodani *et al.*, 2005). The surfactant activities of these peptides are

evidenced by their capacity to restore aerial development to *Streptomyces* mutants blocked in aerial hyphae formation [the so-called bald (*bld*) mutants] when added extracellularly (Willey *et al.*, 1991; Tillotson *et al.*, 1998; Kodani *et al.*, 2005).

The SapB structural gene, *ramS*, encodes a 42-amino-acid peptide. The C-terminal half of the peptide undergoes post-translational modification involving the dehydration of four alanine residues and the introduction of two lanthionine bridges. The modified peptide is exported and the leader peptide is cleaved to yield the 21-amino-acid mature SapB (Kodani *et al.*, 2004; Willey *et al.*, 2006). *ramS* is part of an operon that includes three other genes: *ramC*, which encodes an enzyme thought to dehydrate and cyclize the RamS translation product (Kodani *et al.*, 2004), and *ramA* and *ramB*, which encode components of an ABC transporter likely involved in SapB export (Ma and Kendall, 1994; Keijser *et al.*, 2000; Kodani *et al.*, 2004; Willey *et al.*, 2006). Transcription of the *ramCSAB* gene cluster is positively regulated by the product of the convergently transcribed *ramR* gene, which encodes a response regulator-like protein that binds to the *ramCSAB* promoter region (Keijser *et al.*, 2002; Nguyen *et al.*, 2002; O'Connor *et al.*, 2002; O'Connor and Nodwell, 2005). There is no cognate sensor kinase genetically linked to *ramR*, and nothing is currently known about the regulatory mechanisms that directly activate *ramR* gene expression at the onset of aerial mycelium formation. *bld* mutants fail to make SapB, but there has yet to be a direct connection established between any *bld* gene products and the *ram* gene cluster in *S. coelicolor*. In wild-type *S. coelicolor*, SapB is produced only during growth on rich medium, and it is completely absent during aerial development on minimal medium (Willey *et al.*, 1991).

The chaplins are a group of eight secreted, surface-active proteins that are necessary for aerial hyphae formation (Claessen *et al.*, 2003; Elliot *et al.*, 2003a). All chaplins have an N-terminal Sec secretion signal, and they share a conserved hydrophobic domain called the 'chaplin domain'. Five of the chaplins, ChpD–H, consist solely of a signal peptide and a single-chaplin domain, and are referred to as the 'short' chaplins, while the remaining three 'long' chaplins (ChpA–C) have a signal peptide and two chaplin domains followed by a C-terminal sorting signal that targets them for covalent attachment to the cell wall by the sortase enzyme (Claessen *et al.*, 2003; Elliot *et al.*, 2003a; Marraffini *et al.*, 2006). Deletion of individual chaplin genes has no discernible effect on colony morphology, and at least four chaplin genes must be knocked out before a notable delay in morphogenesis is detectable. Unlike SapB, chaplin production does not appear to be medium dependent, as the chaplin genes are transcribed both on rich medium (Elliot *et al.*, 2003a) and on minimal medium (Claessen *et al.*, 2003). Like

SapB, however, the chaplins are not expressed by *bld* mutants grown on non-permissive rich media (Elliot *et al.*, 2003a) and, as is the case for *ramR*, nothing is known about their upstream regulation. The chaplins and SapB also share substantial surfactant characteristics, both having the capacity to dramatically reduce the surface tension of water (Tillotson *et al.*, 1998; Claessen *et al.*, 2003). *In vivo*, the chaplins are also proposed to contribute to an intricate 'rodlet' ultrastructure found on the surface of aerial hyphae and spores (Claessen *et al.*, 2004), and *in vitro*, the chaplins are capable of self-assembling into insoluble filaments that are reminiscent of amyloid-like fibres (Claessen *et al.*, 2003).

The presence of two separate pathways leading to the erection of aerial hyphae in *S. coelicolor* is suggested by the observation that many *bld* mutants are conditionally defective in their ability to raise aerial hyphae (Kelemen and Buttner, 1998; Nguyen *et al.*, 2002; Chater and Horinouchi, 2003). That is, these *bld* mutants are blocked in aerial hyphae formation when grown on rich medium, but can undergo complete morphological differentiation when grown on minimal medium in which glucose has been substituted with an alternative carbon source such as mannitol or arabinose. SapB production is blocked in all *bld* mutants when grown on rich medium, with the exception of *bldM* and *bldN* (where SapB production is concomitant with delayed aerial hyphae formation; J.M. Willey, unpubl. data). In contrast, SapB is not made by any strain (wild type or *bld* mutant) when grown on minimal medium (Willey *et al.*, 1991). It is thought that the elimination of the cohesive forces between water molecules covering filaments within a mycelium is prerequisite to the emergence of aerial hyphae, thus a different surface active molecule must fulfil the biosurfactant role in the SapB-independent developmental pathway. A strong candidate for this role is the chaplins.

Until now, little was known about the redundancy or functional interplay between SapB and the chaplins. In this study, we demonstrate that SapB and the chaplins overlap in their functional roles of enabling aerial hyphae formation, that the chaplins are key contributors to the 'SapB-independent' pathway of aerial hyphae formation on minimal medium, but that the chaplins and SapB are both required for normal aerial hyphae formation on rich medium.

Results

Deletion of all chaplin genes results in a conditionally bald mutant phenotype

It was previously demonstrated that deletion of four or more chaplin genes results in delayed aerial hyphae formation (Claessen *et al.*, 2003; Elliot *et al.*, 2003a), and
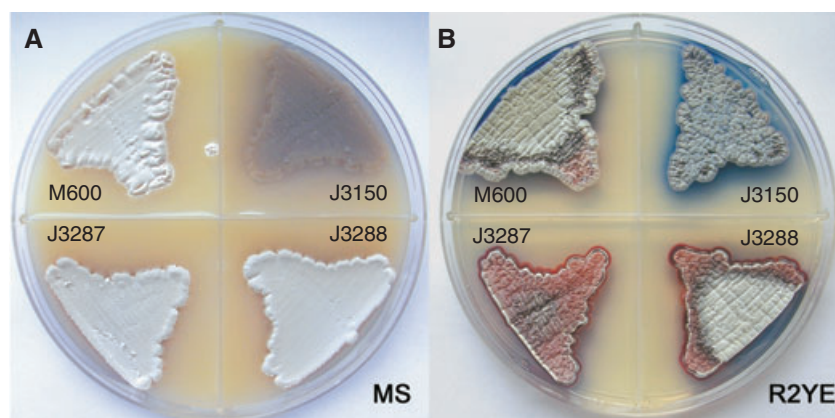
Fig. 1. *ram* mutants and *chp* mutants are differentially affected in their abilities to form aerial hyphae. Phenotypic comparison of Δ*ramR* (J3287), Δ*ramCSAB* (J3288), 8× *chp* mutant (J3150) and their congenic parental strain (M600) grown on (A) MS medium (7 days) or (B) R2YE medium (4 days). Note the obverse defects in aerial hyphae formation of the *ram* mutants versus the *chp* mutant.

that loss of all eight chaplin genes in an *S. coelicolor* M145 genetic background results in severe attenuation of aerial hyphae formation when grown on MS (soy flour mannitol) medium (Claessen *et al.*, 2004). Similarly, we found that deletion of seven or all eight chaplin genes in an M600 genetic background (strains J3149 and J3150 respectively) resulted in a severe morphological defect on MS medium. However, these strains were conditionally bald: they initially appeared bald on MS medium, and were capable of raising only a very sparse aerial mycelium on MS medium after 6–7 days (as opposed to the wild-type strain where aerial hyphae were detectable after only 2 days) (Fig. 1A). In contrast, on rich medium (R2YE), however, the chaplin mutants raised an aerial mycelium with similar growth kinetics to the wild-type strain, but with the overall abundance of hyphae significantly reduced (Fig. 1B). An identical phenotype was observed for chaplin mutants grown on R2YE lacking sucrose (data not shown). As observed previously for the M145 8× *chp* mutant (Claessen *et al.*, 2004), the surface of the aerial hyphae of the M600 8× *chp* mutant was devoid of the rodlet ultrastructure characteristic of the wild type and, despite its lack of aerial hyphae on MS medium, was still capable of making occasional spore chains in prostrate surface hyphae (data not shown). While the primary role of the chaplins in aerial development is predicted to be a structural one, the reduced sporulation observed in the 8× *chp* mutant compared with the wild-type strain suggests there may be additional signalling events that are blocked in the chaplin mutant.

Comparison of chp *and* ram *null mutant phenotypes*

While various *ram* mutant strains have been reported (Keijser *et al.*, 2000; Nguyen *et al.*, 2002; O'Connor *et al.*, 2002), direct comparisons between *chp* and *ram* mutant phenotypes have not been possible because of their differing genetic backgrounds. To address this, we used the *S. coelicolor* M600 wild-type strain, and constructed two

ram mutant strains for comparison with the full 8× *chp* mutant constructed in the same genetic background. In one strain we deleted the *ramCSAB* operon, which includes the SapB structural gene, *ramS*, and in the other we deleted *ramR*, which encodes the *ramCSAB* transcriptional activator. As expected, the SapB-deficient *ramCSAB* and *ramR* mutant strains (J3288 and J3287 respectively) had very similar phenotypes: when grown on rich medium (R2YE), they were initially bald, but formed a substantial aerial mycelium upon extended incubation, with J3288 (Δ*ramCSAB*) developing an aerial mycelium slightly earlier than J3287 (Δ*ramR*) (Fig. 1B). However, when grown on minimal-mannitol or MS agar, they were phenotypically indistinguishable from the wild-type strain (Fig. 1A). This is in contrast to the observations made by O'Connor *et al.* (2002), who report that a *ramC* knockout in an M145 background appears to be developmentally delayed on both rich and minimal-mannitol media, and Keijser *et al.* (2000), who describe a *ramRAB* mutant, also in an M145 background, that was morphologically identical to its wild-type parent regardless of media composition.

The full 8× *chp* mutant (J3150) exhibited an obverse conditional phenotype to that of the *ram* mutants: as described above, on MS medium, it raised a very sparse aerial mycelium only after prolonged incubation, but formed an aerial mycelium on rich medium (R2YE) with wild-type growth kinetics. Thus, J3150 had a conditional bald phenotype, but suppression of the mutant phenotype was contrary to that of the *ram* mutants and the classical *bld* mutants, which appear wild type when utilizing alternative carbon sources and are bald on rich medium.

Overexpression of ramR *restores aerial hyphae formation to a* chp *mutant irrespective of growth media*

Restoration of aerial hyphae formation in most *bld* mutants can be achieved by overexpressing *ramR*, which
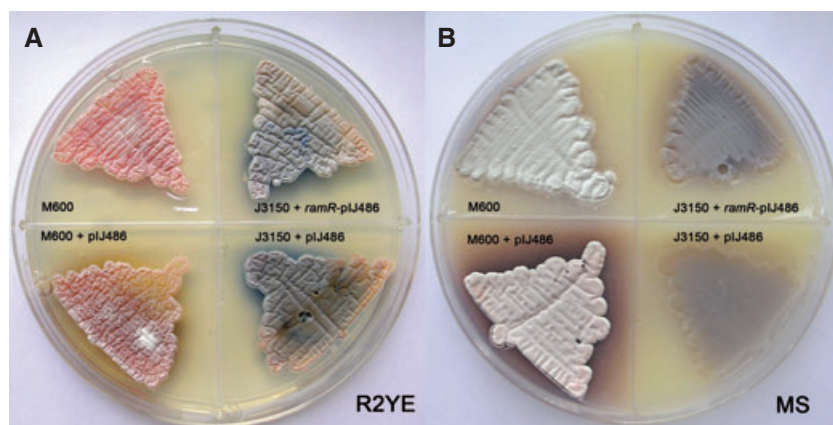
Fig. 2. Overexpression of *ramR* induces aerial hyphae formation in J3150, the 8× *chp* mutant. *ramR* cloned into the multicopy *Streptomyces* vector pIJ486 increased aerial hyphae abundance of J3150 on both (A) rich R2YE medium (3 days) and (B) MS medium (7 days) relative to J3150 containing pIJ486 alone. Shown for comparison are the wild-type M600 and M600 containing pIJ486.

drives unregulated overproduction of SapB such that SapB is produced on minimal medium by wild-type strains (Nguyen *et al.*, 2002). Given the conditional bald phenotype of the 8× *chp* mutant (J3150), we were interested to determine if *ramR* overexpression could also restore wild-type levels of aerial hyphae formation when this strain was grown on a variety of media/carbon sources. *ramR* was placed on a high-copy-number plasmid (pIJ486), and was introduced into J3150. Multiple copies of *ramR* in J3150 grown on rich medium (R2YE) restored the abundance of aerial hyphae to near wild-type levels (Fig. 2A). Interestingly, high-copy *ramR* also restored a modest level of aerial hyphae formation to J3150 when grown on MS medium (Fig. 2B). This suggested that the defects in aerial development exhibited by the *chp* mutant could be partially overcome by the overproduction of a molecule under RamR control, with SapB the most likely candidate.

SapB application rescues both ram *and* chp mutant phenotypes

Additional evidence that SapB could mediate aerial hyphae formation in the absence of either endogenously synthesized SapB or the chaplins was obtained through extracellular complementation experiments using exogenous addition of SapB. It is well established that exogenously applied SapB restores the capacity of *bld* mutants to form aerial hyphae (Willey *et al.*, 1991) by releasing undifferentiated hyphae from the aqueous colony surface (Tillotson *et al.*, 1998). To determine whether the application of SapB to either a *ramCSAB* or a *ramR* mutant had the same effect, the *ram* mutants were spread on R2YE or MS media. SapB was applied to the surface of each mutant strain, and identical results were seen after 2 days of growth: zones of aerial differentiation (hyphae, and later spores) were visible in the spots where SapB had been applied, while the rest of the mycelium remained bald (Fig. 3A). These results are similar to those obtained when SapB is applied to a *ramS* null strain (Kodani *et al.*, 2005). When a similar experiment was performed on MS medium to test the response of the chaplin-deficient strain (J3150), a robust aerial mycelium developed where SapB was applied, again after 2 days of growth (Fig. 3B). In all instances, resultant aerial hyphae gave rise to spore
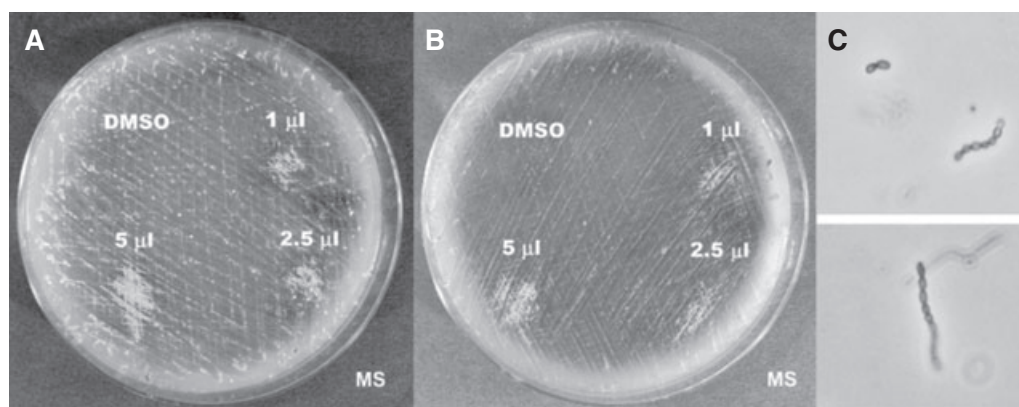

Fig. 3. A and B. Exogenous application of purified SapB accelerates aerial hyphae formation of (A) J3288 (Δ*ramCSAB*) and (B) J3150 (Δ8× *chp*) on MS medium after 3 days of growth. For each strain, 1, 2.5 and 5 μl aliquots of purified SapB (0.5 μg μl⁻¹) were applied to the surface after 2 days of growth. DMSO (5 μl) was applied as a negative control. C. Coverslip impressions taken after exogenous application of SapB to J3150 show sporulating aerial hyphae.

Table 1. Correlation between aerial hyphae formation and chaplin/SapB detection for wild type (M600) and the *chp* and *ram* null mutant strains.

	Day						
	1	2	3	4	5	6	
M600							
R2YE	–	+	++	++	++	++	Aerial hyphae
			+C; +S	+C; +S	+C; +S	+C; +S	Chp/SapB observations
MS	–	+	++	++	++	++	Aerial hyphae
			+C; –S	–S	–S	+C; –S	Chp/SapB observations
Δ8× *chp*							
R2YE	–	+	+	+	+	+	Aerial hyphae
				+S	+S	+S	Chp/SapB observations
MS	–	–	–	–	–	+	Aerial hyphae
				–S	–S	+S	Chp/SapB observations
Δ*ramR*/Δ*ramCSAB*							
R2YE	–	+	+	+	++	++	Aerial hyphae
			+C	+C	+C	+C	Chp/SapB observations
MS	–	+	+	++	++	++	Aerial hyphae
							Chp/SapB observations

Aerial hyphae: –, none; +, sparse; ++, abundant.
+C, chaplins detected by MALDI-ToF mass spectrometry; +S, SapB detectable by Western blot; –S, no SapB detectable by Western blot.

chains, as detected through coverslip impressions (Fig. 3C). This suggested that the 8× *chp* mutant lacked only sufficient surfactant molecules to raise aerial hyphae, and that the addition of SapB overcame both this deficiency, and any signalling block associated with knockout of all chaplin genes. Taken together with the *ramR* overexpression results, this suggests that SapB, and not some other molecule under RamR control, can drive aerial hyphae formation in the absence of chaplins.

There is a direct correlation between aerial hyphae formation and the production of the chaplins/SapB

Given the apparent ability of purified SapB to fulfil the role of biosurfactant in the absence of the chaplins, and considering the reciprocal phenotypes of the *ram* and *chp* mutant strains, we were curious to determine whether the conditional bald phenotypes of each mutant strain could be correlated with production of the remaining surfactant molecules (either the chaplins or SapB). Using the wild-type M600 strain and the congenic derivatives J3288 (Δ*ramCSAB*), J3287 (Δ*ramR*) and J3150 (8× *chp* mutant), we followed the production of the five short chaplins (ChpD–H) and SapB over 6 days using MALDI-ToF mass spectrometry and immunoblot analysis respectively. The wild-type

strain demonstrated very similar growth kinetics on both rich (R2YE) and MS media, with aerial hyphae starting to appear 2 days after inoculation, and sporulation initiating after 3 days. Cell surface extracts were collected from strains at 24 h intervals, beginning 2 days after inoculation. In the wild-type strain, chaplin proteins could be detected after 3 days of growth, and were still present in sporulating cultures after 7 days on both rich (R2YE) and MS media (Table 1). We also followed the surface localization of the chaplins in the two *ram* mutant strains when they were grown on R2YE medium. After 2 days, few aerial hyphae could be detected, but at 3 days there was a substantial increase in aerial hyphae abundance, and this coincided with measurable chaplin protein production (Table 1). Thus, the timing of chaplin production in the *ram* mutant strains, as well as chaplin abundance, appeared to be identical to the wild-type strain on rich medium.

Immunoblot analysis was performed to explore SapB production in the three mutant strains. Predictably, the *ram* null mutant strains failed to make SapB (data not shown), while J3150 (8× *chp* mutant) produced greater than wild-type levels of the peptide on rich medium (Fig. 4). Intriguingly, J3150 also produced SapB when grown for an extended period on MS medium, with SapB production concomitant with the appearance of sparse
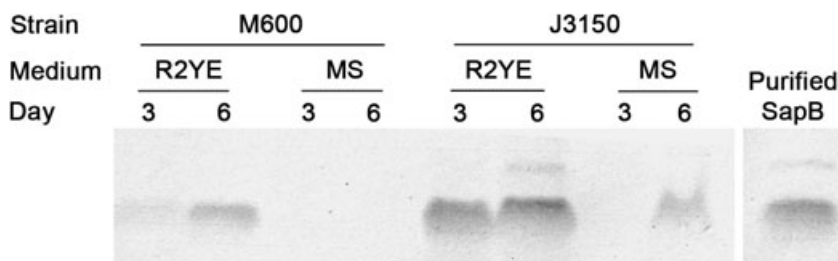

Fig. 4. Immunoblot analysis of SapB isolated from aerial hyphae and spores of wild-type M600 and 8× *chp* mutant J3150 after 3 or 6 days of growth on both R2YE and MS media. Unexpectedly, J3150 produced SapB after 6 days of growth on MS medium, in contrast to all previously examined *S. coelicolor* strains.
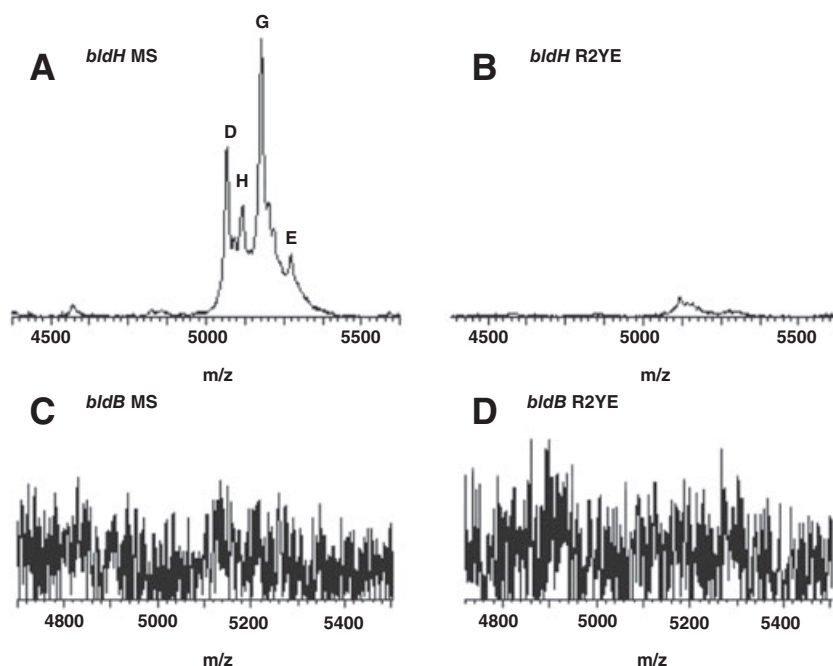
aerial hyphae (Fig. 4). The capacity of the 8× *chp* mutant to produce SapB on a medium where its synthesis is normally suppressed suggests that the repression of *ramR* expression seen in wild-type strains can be partially alleviated in a *chp* mutant background.

Conditional bld *mutants make the chaplins on mannitol-containing media*

The agents involved in the 'SapB independent' pathway of aerial hyphae formation have not been identified, but given the opposing *ram* versus *chp* mutant phenotypes, it was logical to consider the chaplins as likely candidates. To examine this possibility, we tested an assortment of *bld* mutants for chaplin production on rich (R2YE) and MS media. Based on previous RNA analyses (Elliot *et al.*, 2003a), we knew that at least two of the chaplin genes were very poorly expressed in a number of *bld* mutants (*bldN*, *bldM*, *bldH*, *bldG* and *bldC*) when grown on rich medium. What was not known, however, was whether the chaplins were expressed when *bld* mutants differentiate on MS medium, and whether chaplin expression could be in any way correlated with the appearance of aerial hyphae in the conditional *bld* mutants.

Several *bld* mutants (in various genetic backgrounds) were examined for chaplin gene expression, including *bldH*, *bldD* and *bldA* (conditional mutants that sporulate when grown on MS medium), as well as *bldB*, which is unable to raise aerial hyphae on any medium. The mutants were grown on R2YE and MS media for 7 days, after which cell surface proteins were isolated, and the presence or absence of chaplin proteins was assessed.

As shown in Fig. 5A and B, peaks corresponding to the chaplin proteins were negligible in the extract from the R2YE-grown culture of *bldH*, but were readily detectable in the extract from the MS-grown culture. Identical results were obtained for *bldD* and *bldA* (data not shown). In contrast, chaplins were not detectable in any extract isolated from a *bldB* mutant, irrespective of the growth medium (Fig. 5C and D), showing a correlation between aerial mycelium formation and chaplin production.

A complete chp/ram *mutant is bald on all media*

Given the contrasting phenotypes of the *chp* and *ram* mutant strains on rich and MS media, the 'SapB-dependent' and 'SapB-independent' pathways for aerial hyphae formation, and the similar surfactant capabilities of SapB and the chaplins, we wanted to determine whether deletion of both the *chp* and the *ram* genes would result in a mutant strain unable to raise an aerial mycelium on any medium (a *bldB*-like unconditional phenotype). A full *chp/ram* mutant was created using a viomycin resistance cassette to replace either *ramR* (J3289) or the *ramCSAB* (J3290) gene cluster in the 8× *chp* mutant background. Immunoblot and MALDI-ToF analyses confirmed that these strains produced neither SapB nor the chaplins (data not shown). The *chp/ram* null mutant strains failed to raise any aerial hyphae on MS medium (a phenotype similar to that of the 8× *chp* mutant), and were only capable of raising an extremely sparse aerial mycelium on rich R2YE medium after extended incubation (Fig. 6). These sparse aerial hyphae were undetectable by coverslip impressions, and unlike typical aerial struc-
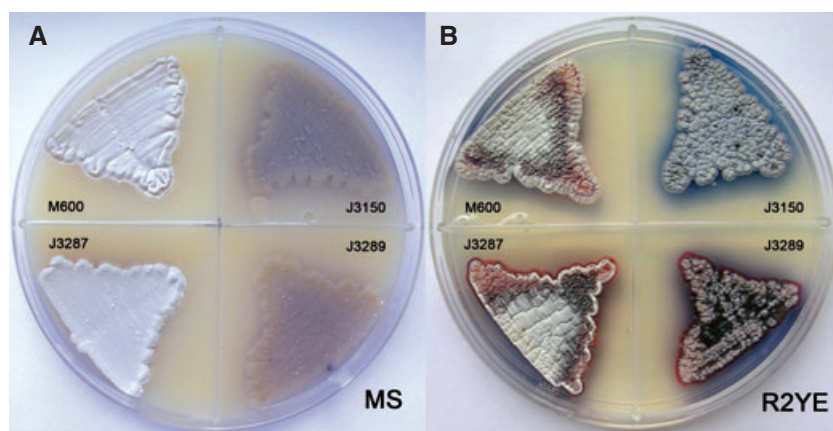
Fig. 6. Phenotype of a full *chp/ram* mutant strain (J3289), relative to J3150 (8× *chp* mutant), J3287 (*ramR* null mutant) and M600 (wild type). The full *chp/ram* mutant makes (A) no detectable aerial hyphae on MS medium after 7 days, and (B) few hyphae on R2YE medium after 4 days.

tures, were highly hydrophilic (data not shown). These results suggest that aerial hyphae formation requires the activity of either the chaplins (MS medium) or SapB and the chaplins (rich medium), and in the absence of both molecules, a robust aerial mycelium fails to develop on all media tested.

Discussion

Surfactant requirement is medium specific

Many *bld* mutants are defective in both aerial hyphae formation and SapB production when grown on rich medium, but are able to raise an aerial mycelium in the absence of SapB on minimal media. This has brought about the idea of two separate pathways leading to the erection of aerial hyphae: a *bld* gene/SapB-dependent pathway and a *bld* gene/SapB-independent pathway (Kelemen and Buttner, 1998; Nguyen *et al.*, 2002; Chater and Horinouchi, 2003). Both SapB (Tillotson *et al.*, 1998) and the chaplins (Claessen *et al.*, 2003; Elliot *et al.*, 2003a) are believed to act as surfactants during aerial morphogenesis, reducing the surface tension at air–aqueous interfaces, enabling nascent hyphae to grow into the air. The chaplins are also localized to the surface of aerial hyphae and spores where they could act to prevent the desiccation of aerial filaments following emergence from the aqueous vegetative environment. This would be consistent with a model for chaplin activity put forward by Chater and Chandra (2006), where they propose a role for the chaplins in maintaining an aqueous environment for cellular development during aerial hyphal growth. The observation that *ram* mutants (and thus SapB mutants) are not fully bald but are merely delayed in aerial hyphae formation on rich medium indicates that SapB is not the only molecule capable of facilitating aerial hyphae development under these conditions. Our results suggest that growth of the aerial mycelium on rich medium is dependent on both the chaplins and SapB. In contrast, aerial

mycelium formation during SapB-independent morphogenesis on minimal medium appears to require only the chaplins, where they were produced by both conditional *bld* mutants and wild-type strains. The direct correlation between chaplin production on minimal media and aerial hyphae formation is strengthened by the observation that the non-conditional *bld* mutant, *bldB*, was blocked in chaplin production under all conditions examined.

Regulating the production of surfactant molecules

The obverse phenotypes of *ram* and *chp* mutants, and the differing expression profiles of SapB and the chaplins, reveal a sophisticated regulatory network governing surfactant production in *S. coelicolor*. These regulatory differences have resulted in two separate, but not completely independent pathways of aerial hyphae formation: the SapB-dependent pathway of aerial hyphae formation on rich (R2YE) medium, to which the chaplins contribute, and the chaplin-dependent pathway (or SapB-independent pathway) of aerial hyphae formation on MS medium, to which SapB makes no contribution (Fig. 7). The production of both SapB and the chaplins on rich medium requires the *bld* genes; however, it appears that chaplin expression on MS medium is largely *bld* gene independent, as conditional *bld* mutants produce significant levels of chaplin proteins on MS medium, and are consequently able to raise a substantial aerial mycelium.

In *S. coelicolor*, the *ramCSAB* operon is positively controlled by RamR (Keijser *et al.*, 2002; Nguyen *et al.*, 2002; O'Connor *et al.*, 2002; O'Connor and Nodwell, 2005). In *Streptomyces griseus*, the orthologous *amfTSAB* operon (Ueda *et al.*, 1993) is similarly governed by the RamR orthologue, AmfR, but is negatively regulated by BldD (Ueda *et al.*, 2005). *ramCSAB* regulation by BldD does not, however, appear to be conserved in *S. coelicolor* (B.K. Leskiw and J.R. Nodwell, pers. comm.). While *ramR*
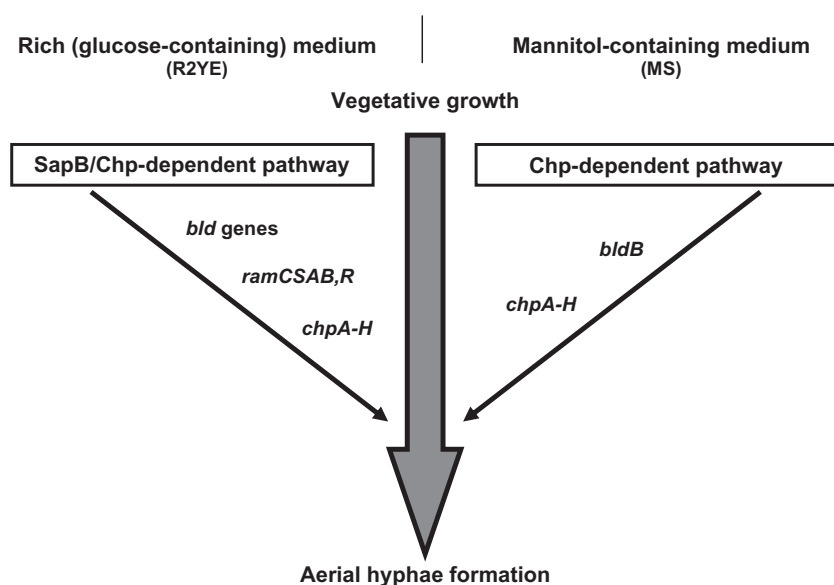
Rich (glucose-containing) medium (R2YE)	Mannitol-containing medium (MS)

Vegetative growth

SapB/Chp-dependent pathway	Chp-dependent pathway

bld genes

ramCSAB,R

chpA-H

bldB

chpA-H

Aerial hyphae formation

Fig. 7. Regulation of aerial hyphae formation in *S. coelicolor*. Two separate pathways allow for aerial hyphae formation in *S. coelicolor*. On rich medium, aerial hyphae formation requires the activity of the *bld* genes, as well as that of SapB and the chaplins, while on minimal medium, only the chaplins are essential, with neither SapB nor the majority of the *bld* genes (with the exception of *bldB*) being necessary.

gene expression is obviously sensitive to media composition (repressed on minimal and MS media), the direct regulators of this phenomenon in *S. coelicolor* are unknown. Similarly, there is currently nothing known about the direct regulators of chaplin gene expression.

One particularly intriguing observation is that the 8× *chp* mutant ultimately produces SapB on MS medium, enabling it to raise a sparse aerial mycelium. As wild-type strains do not produce SapB on MS medium, it is clear that the repression of *ramR* expression seen on MS medium in wild-type strains is alleviated in the 8× *chp* mutant. This may represent a compensatory response to the absence of normal chaplin/surfactant production under these conditions. Similarly, the production of SapB in the 8× *chp* mutant on rich (R2YE) medium is increased relative to the wild type, likely contributing to the formation of the observed aerial mycelium. Gaining insight into the mechanism by which the normal regulation of *ramR* can be overridden under these circumstances presents an additional challenge for the future.

Multiple surfactants in the streptomycetes and filamentous fungi

It is apparent from this work that SapB and the chaplins fulfil very similar roles in *Streptomyces* development, and that contributions by both are essential for aerial hyphae formation under particular conditions. Their importance is underscored by the conservation of the *ram* and *chp* genes in other *Streptomyces* species (Elliot *et al.*, 2003a; Willey *et al.*, 1991; 2006). A question that remains unanswered is why streptomycetes have evolved and maintained two distinct, yet functionally overlapping surfactant systems, with one used in a nutrient-poor environment,

and both used in a nutrient-rich environment. Sporulation is the primary means of reproduction and dispersal for *Streptomyces*, and biosurfactant production is necessary for this process to occur efficiently in solid culture. Given that soil is a highly variable environment, it seems likely that streptomycetes have evolved the capacity to produce surfactant molecules that are optimized for different environmental conditions.

It is conceivable that, in addition to SapB and the chaplins, there are as yet undiscovered surfactants that make a minor contribution to aerial hyphae formation. This possibility is strengthened by San Paolo *et al.* (2006), who identified a new RamR-regulated gene cluster that, when overexpressed, stimulates aerial hyphae formation in a SapB-independent, and likely chaplin-independent, manner.

Like *Streptomyces*, the filamentous fungi raise aerial structures with the aid of surfactant molecules termed hydrophobins (as reviewed by Wösten, 2001). A number of fungi have multiple hydrophobin genes; however, the resulting proteins are usually differentially expressed and have distinct functions, rather than overlapping functions as seen for SapB and the chaplins. The production of partially redundant surfactant species is not unprecedented, however, as the fungus *Schizophyllum commune* produces several different hydrophobins (Wessels *et al.*, 1991), as well as an unrelated protein that can mediate the emergence of aerial hyphae (Lugones *et al.*, 2004). In addition, the plant pathogen *Ustilago maydis* raises aerial hyphae through the activity of proteins unrelated to the hydrophobins, termed 'repellants' (Wösten *et al.*, 1996). This implies that, like streptomycetes, filamentous fungi can use different surfactant molecules to raise aerial structures.

Table 2. *Streptomyces coelicolor* strains.

Strain	Genotype	Reference
M600	SCP1⁻ SCP2⁻	Chakrabartty and Bibb (1997)
J1501	*hisAI uraAI strAI* Pgl⁻ SCP1⁻ SCP2⁻	Chater *et al.* (1982)
J3150	M600 ΔchpAD ΔchpCH::aadA ΔchpB ΔchpF ΔchpG ΔchpE::apr	This work
J3287	M600 ΔramR::vph	This work
J3288	M600 ΔramCSAB::vph	This work
J3289	M600 ΔchpAD ΔchpCH::aadA ΔchpB ΔchpF ΔchpG ΔchpE::apr ΔramR::vph	This work
J3290	M600 ΔchpAD ΔchpCH::aadA ΔchpB ΔchpF ΔchpG ΔchpE::apr ΔramCSAB::vph	This work
C109	J1501 *bldH*	Champness (1988)
J1700	J1501 *bldA39*	Piret and Chater (1985)
ΔbldD	M600 *bldD::apr*	Elliot *et al.* (2003b)
J669	*bldB43 mthB2 cysD18 agaA7* SCP1ᴺᶠ SCP2*	Merrick (1976)

Surfactants and aerial development

If the importance of SapB and the chaplins in the erection of aerial hyphae in *S. coelicolor* were exclusively due to their surfactant properties, then it might be expected that any surfactant could substitute for them and fulfil this role. Supporting this prediction is the observation that the *S. tendae* lanthionine-containing peptide, SapT (Kodani *et al.*, 2005) and the hydrophobin protein SC3 from the fungus *S. commune* (Tillotson *et al.*, 1998) are able to stimulate aerial hyphae formation in *S. coelicolor bld* mutants when applied exogenously. In contrast to this, however, is the finding that the *Bacillus subtilis* surfactant peptide surfactin (Richter *et al.*, 1998), which is required for development of aerial structures in *B. subtilis*, strongly inhibits aerial hyphae formation when applied to *S. coelicolor*, without apparent detriment to vegetative filaments (Straight *et al.*, 2006). Intriguingly, the surfactin-mediated block in aerial development is alleviated by the addition of either purified SapB or the chaplins (Straight *et al.*, 2006). These results demonstrate that microbial surfactants are not necessarily interchangeable, and that they exhibit specificity in their mode of action. They also suggest that in natural microbial communities, such compounds may have functions beyond their obvious roles as surface active molecules. We may therefore not fully understand the roles of SapB and the chaplins until they are analysed in more natural, complex microbial populations.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Streptomyces strains (Table 2) were cultured at 30°C on R2YE, MS, minimal and DNA agar media, or in a 1:1 mixture of tryptone soya broth (TSB) and yeast extract-malt extract (YEME) supplemented with MgCl₂ and glycine liquid media (Kieser *et al.*, 2000). *Escherichia coli* strain BW21153/pIJ790 was grown at 30°C and was used for PCR-targeted gene disruptions (Datsenko and Wanner, 2000; Gust *et al.*, 2003), while DH5α (used for plasmid construction and routine subcloning) and ET12567/pUZ8002 (used for generation of methylation-free DNA; Kieser *et al.*, 2000) were grown at 37°C. Plasmids used were pGEM-T (Promega), pIJ2925 (Bierman *et al.*, 1992), pIJ486 (Ward *et al.*, 1986) and pKN22 (Nguyen *et al.*, 2002), which is a derivative of pHM11a (Motamedi *et al.*, 1995) containing *ramR* under the control of the constitutive *ermE* promoter.

Construction of chp and/or ram null mutants

Null mutants were constructed as described in Elliot *et al.* (2003a) according to the methods of Gust *et al.* (2003). The construction of a sevenfold (7×) and an eightfold (8× – complete chaplin knockout) *chp* mutant strain followed on from the fivefold *chp* mutant (J3145) created by Elliot *et al.* (2003a), where *chpAD* was replaced with an apramycin resistance cassette which was then removed to generate an in-frame deletion (and to allow recycling of the apramycin resistance cassette), *chpCH* was replaced by a spectinomycin/streptomycin resistance cassette, and *chpB* was replaced with an apramycin resistance cassette. The apramycin resistance cassette was then removed from *chpB* by a second targeting event, to generate J3145A. As *chpF* and *chpG* are separated by only six genes, they were both knocked out of cosmid SCC61A (*chpF* was first knocked out using an apramycin resistance cassette, which was then removed and used to replace *chpG*), and both mutations were introduced simultaneously into the fivefold mutant, creating J3149. The apramycin resistance cassette was then removed from *chpG* (to create J3149A, the 7× mutant), and was used to replace *chpE* on cosmid I5, which was then introduced into the 7× mutant by conjugation to create the 8× mutant (J3150).

Null mutants in the *ram* gene cluster were constructed by replacing the entire *ramR* gene or *ramCSAB* gene cluster with a cassette carrying the viomycin resistance gene (*vph*) and *oriT* from RK2 by PCR targeting (Gust *et al.*, 2003). Cosmid SC6G3 was introduced into *E. coli* BW21153 carrying plasmid pIJ790. *ramR* and *ramCSAB* were disrupted separately by electroporation of the cells with the PCR-amplified *vph-oriT* cassette, generated using primers ramR Fwd and ramR Rev, or ramC Fwd and ramB Rev (Table 3). The resulting cosmid, carrying the viomycin resistance cassette in place of either *ramR* or *ramCSAB*, was then introduced into the methylation-deficient *E. coli* ET12567 carrying

Table 3. Oligonucleotide sequences used in this study.

Oligo name	Sequence (5′ to 3′)
ramR Fwd	GCCCGGTGGCCCGAACGGGGCCGCCGCTGACAAGGGGTGATTCCGGGGATCCGTCGACC
ramR Rev	ACCGGGACCTGACGGGACACTGGAACTCGTGACGGGTCATGTAGGCTGGAGCTGCTTC
ramC Fwd	CCGCGAAGTTACTTGTGCGTTCCGCACCTCGGAGTGGTGATTCCGGGGATCCGTCGACC
ramB Rev	TCCGCATCGTGCAGAGCGCCGGCTGGACCTGACCCGTCATGTAGGCTGGAGCTGCTTC
ramR for	CGAAGACGGCGGACTCCCGTC
ramR down	CGAGGCCCGCGCGGAGAAGG
486 oligo	CATGCGAAACGATCCTCATCC
Fd univ	CGCGTGATCAGCCCCGCAAAAGCGGCCTTTGAC

pUZ8002 before being transferred into *S. coelicolor* M600 or J3150 by conjugation (Kieser *et al.*, 2000). Viomycin-resistant, kanamycin-sensitive exconjugants were identified, isolated, and confirmed by PCR and Southern blot hybridization (see Table 2). Complementation of the *ramR* mutant was achieved using the plasmid pTO11 (O'Connor *et al.*, 2002), while the *ramCSAB* mutant was complemented by the introduction of the *ramCSAB* gene cluster on plasmid pIJ6902 (Huang *et al.*, 2005). The *ramCSAB* cluster was excised from cosmid 6G3 using EcoRI and MscI, before being cloned into pBluescript KS (Stratagene) digested with EcoRI and EcoRV. The cluster was then excised using EcoRI and XhoI, and was cloned into pIJ6902 digested with the same enzymes.

Construction of the ramR *overexpression plasmid*

ramR was PCR amplified using *S. coelicolor* M600 chromosomal DNA as template, Expand High Fidelity DNA polymerase, and primers ramR for and ramR down (Table 3). The resulting product was introduced into pGEM-T (Promega), and *ramR* clones were verified by digesting with SphI and SpeI. Using these enzymes, *ramR* was excised, cloned into pIJ2925 digested with SphI and XbaI, and the correct insertion was confirmed by PCR. pIJ2925+*ramR* was then passaged through *E. coli* ET12567, to generate methylation-free DNA. Non-methylated *ramR* was excised as an EcoRI and HindIII fragment and was cloned into EcoRI- and HindIII-digested and dephosphorylated pIJ486 before the entire ligation reaction was introduced into *S. coelicolor* J3150 by protoplast transformation (Kieser *et al.*, 2000). Thiostrepton was used for positive transformant selection. Correct constructs were verified by PCR using primers 486 oligo and Fd univ (Table 3). As a negative control, pIJ486 was also introduced into wild-type *S. coelicolor* M600 and the 8× *chp* mutant J3150 by protoplast transformation, and thiostrepton-resistant transformants were selected.

Purification of SapB and immunoblot analysis

Extraction of cellular proteins from *S. coelicolor* strains and the detection of SapB production by immunoblot were performed as described previously (Guijarro *et al.*, 1988). Purified SapB, used as a positive control for the immunoblots and for the exogenous application to lawns of J3150, J3287 and J3288, was isolated from spores of *S. coelicolor* J1501/pKN22, as reported in Kodani *et al.* (2004).

Purification and analysis of the chaplin proteins

The chaplin proteins were isolated from cell wall extracts as described previously (Elliot *et al.*, 2003a), with several modifications. *S. coelicolor* wild-type and mutant strains were grown on cellophane discs on the surface of MS or R2YE solid media. After 2–7 days, cells were scraped from the surface of the cellophane, and were re-suspended in 10 ml of Tris buffer (pH 8.0). The suspension was sonicated on ice for 3–15 s before spinning at 8000 *g* for 10 min. The supernatant was removed, the remaining pellet was re-suspended in 3 ml of 2% SDS and was heated to 100°C for 10 min. After cooling briefly, the suspension was centrifuged at 8000 *g* for 10 min, and the SDS-heat treatment was repeated. The suspension was transferred to 2 ml Eppendorf tubes, and was spun at 16 000 *g* before discarding the supernatant and flash-freezing the pellet in an ethanol-dry ice bath and drying it by rotary evaporation. Trifluoroacetic acid (400–800 µl) was added to the dried pellet, and the soluble fraction removed and dried using a stream of air. MALDI-ToF mass spectrometry conditions were as described in Elliot *et al.* (2003a).

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