Characterization of regulatory pathways controlling morphological differentiation in *Streptomyces coelicolor*

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Salvatore San Paolo

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Klingelbergstrasse 70

4056 Basel

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auf Antrag von

Prof C. Dehio, Prof. U. Jenal and Prof. C. J. Thompson
(Mitglieder des Dissertationskomitees)

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Prof. H. J. Wirz
Dekanin/Dekan

(Name des/der amtierenden
Dekanin/Dekans einsetzen)

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INTRODUCTION

Cellular physiology requires sensing and adapting to changing conditions in the ecosystem. This ability is particularly well developed in bacteria. Strict coordination of metabolic processes with the environment allows maximization of the growth rate and ensures bacterial survival. Sensory systems assessing the environmental state elicit responses which are integrated with cellular metabolism. This adaptation process constantly occurs throughout bacterial growth and usually involves only fine changes in physiology. However, bacterial growth by necessity will eventually bring about a more severe environmental (nutritional) challenge, which induces broad physiological and morphological changes. In this case, the response is accompanied by morphological differentiation of new cell types, suitable to perform specialized functions that will ensure survival and dispersion of the species in the environment. Thus, investigating the mechanisms underlying these differentiation processes is of fundamental importance to understand the biology of the cell.

The Gram-positive soil-dwellers *Eubacteria* of the genus *Streptomyces* are well suited for such investigations.

The Developmental Biology of *Streptomyces*

Streptomycetes are non-motile pluricellular filamentous bacteria, belonging to the family of *Actinomycetales.* They possess a genome with a high GC content and are characterized by a mould-like mycelial structure, suitable to colonize organic soil debris and soil particles. *Streptomycetes* are aerobic saprophityc bacteria, endowed with a wide repertoire of extracellular hydrolytic enzymes, able to break down complex polymers, such as cellulose, maltose, starch, chitin, xylans, polyphenols, etc…The complexity of the terrestrial ecosystems, where they dwell, reflects, therefore, in a complex life cycle in which morphological differentiation is highly coordinated with physiological changes throughout development, allowing them to adapt to diverse environmental conditions (Chater, 1998; 1993). Such physiological changes result in production of secondary metabolites, some of which have antibiotic activity and/or are pigmented, and are associated with spore germination, vegetative mycelium growth, aerial hyphae formation and finally sporulation. The developmental program of *Streptomycetes* begins with the germination of an exospore, which, in response to unidentified signals, triggers the influx of water, resulting in an increase in size and decreased phase brightness, followed by the emergence of a germination tube (Ensign, 1978) (Fig. 1). This elongates through the incorporation of cell wall material at the apical tips, where occasional branching occurs, generating a dense interconnected network of long filaments (hyphae) or vegetative mycelium (first rapid growth phase). However, unlike unicellular bacteria, where chromosome segregation is followed by cell division, *Streptomyces* hyphae remain associated in multinucleoid syncytial compartments which are only irregularly separated by often incomplete septa. After development of vegetative mycelium (24-34 h in *S. coelicolor*), colony appears translucent and biomass accumulation arrests transiently. Referring, in particular, to *Streptomyces coelicolor* life cycle, an initial physiological differentiation can be already observed at this phase of development. Transmission electron microscopy has, in fact, revealed net glycogen deposition (phase I deposits) in the most superficial region of the vegetative mycelium, where growth of new specialized aerial cells will take place (Chater, 1998). Similar to the germination process, unidentified signals trigger the initiation of aerial growth (Fig 1) and differentiation of a new tissue-like structure (aerial hyphae or aerial mycelium) which confer a white fuzzy appearance to the colony. Although aerial hyphae grow at their tips as multinucleoid cells, unlike vegetative hyphae, they do not branch but switch to a typical

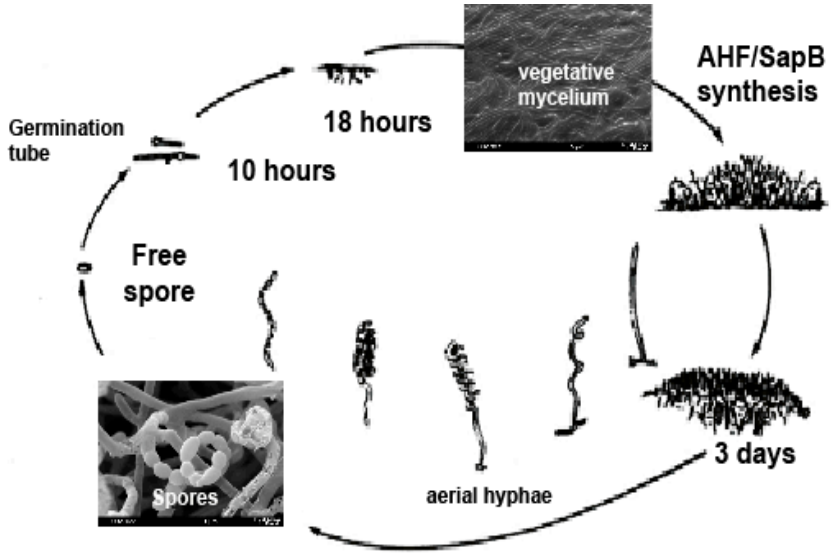

Fig. 1. *Streptomyces coelicolor* life cycle.

coiling-mode of growth (Fig 1). Aerial hyphae formation (AHF), like spore germination, involves the influx of water in order to generate enough turgor pressure to erect hyphae into the air. This may be generated by breaking down polymers such as fats, polyphosphates or glycogen to increase the concentration of osmotically active solutes. These may also serve as nutrients for the differentiation process of erecting aerial hyphae, which eventually will develop into spores. Further nutritional and energetic support to "parasitic" aerial hyphae is supplied by the lysis of the vegetative hyphae in the inner part of the colony (cannibalism). Thus, after the first phase of rapid growth, biomass accumulation not only arrests but even decreases (Transition phase), to be reassumed later with the onset of aerial hyphae formation (second rapid growth phase) (Süsstrunk *et al.,* 1998). Erection of aerial hyphae requires, furthermore, secretion of specific amphipathic compounds (e.g SapB and chaplins in *S. coelicolor*; AmfS in *S. griseus*; streptofactin in *S. tendae*). These function as biosurfactants to breach the surface tension at the air-colony interface, facilitating the emergence of aerial hyphae from the embedded vegetative mycelium (Tillotson *et al.,* 1998; Talbot *et al.,* 2003; Wösten and Willey, 2000). Thus, these bioactive compounds are believed to serve a structural role, rather than cell-cell signals during morphogenesis.

Aerial hyphae formation coincides with the biosynthesis of secondary metabolites (e.g. antibiotics) in the vegetative mycelium. As cell cannibalism and antibiotic biosynthesis take place only in the vegetative mycelium, the developmental process of *Streptomyces* is not only under temporal but also spatial control, like a eukaryotic tissue (Chater, 1998). It was argued that such physiological differentiation between vegetative mycelium and aerial hyphae may serve as a way to protect the colony from microorganisms which may scavenge the nutrients destined to nourish the reproductive phase of the colony. This phase corresponds to the sporulation process, which takes place exclusively in the apical part of mature aerial hyphae and provides further evidence to the spatial control in the *Streptomyces* developmental program. Sporulation involves the conversion of the aerial hyphae into spore chains (Fig 1). This process is initiated by proper partitioning of haploid chromosomes at regular intervals within the hypha. These cytoplasmic regions are, hence, separated into unigenomic compartments through a synchronous septation occurring upon the ladder-like assembly of the cell division protein FtsZ at regular intervals (Schwedock *et al.,* 1997). During sporulation septation, deposits of glycogen (phase II glycogen) are transiently stored in the apical compartments of aerial hyphae. These may serve to supply energy during spore maturation as well as precursors for threhalose stores that will confer osmoprotection to the mature spores and provide them with energy and carbon source during

germination. In a later phase of spore maturation, the cell wall of the prespore compartments mature by thickening, synthesizing a polyketide gray pigment and rounding off into exospores. However, unlike the endospores formed by *Bacillus subtilis*, *Streptomycetes* spores are not resistant to treatment with chemicals, UV and heat. They are, indeed, resistant to desiccation and physical forces (Ensign, 1978), and are covered by several hydrophobic layers, which are believed to facilitate their dispersion in the environment (Wildermuth *et al.,* 1971).

The *Streptomyces* life cycle, described above, refers to development on agar medium. Indeed, in liquid cultures, *Spreptomycetes* undertake a different developmental program. Although aerial hyphae are not differentiated and some species, such as *Streptomyces coelicolor* do not sporulate, development of liquid cultures is characterized by a diauxic growth curve, where we can distinguish four developmental phases, similarly to solid cultures (Fig 2). After an initial phase of rapid growth (RG1, corresponding to vegetative mycelium on solid cultures), a transitory slowdown in growth provides a transition phase (T-phase) to a second period of rapid growth (RG2, corresponding to AHF on solid cultures) and finally to stationary phase (S phase or sporulation on agar medium) (Puglia *et al.,* 1995; Vohradsky *et al.,* 2000). The T-phase on both solid (Fig 1) and liquid cultures (Fig 2) represents a developmental checkpoint for environmental and physiological signals that will lead to the activation of the differentiation program. This phase has been associated with the activation of antibiotic biosynthetic genes (Holt *et al.,* 1992) and regulatory elements needed for antibiotic-induced expression of a multidrug resistance gene (Salah-Bey *et al.,* 1995). The T-phase, furthermore, has been related to the stringent response, as indicated by the accumulation of ppGpp (Holt *et al.,* 1992) and to the stationary phase because of the decreases in the rates of synthesis of ribosomal proteins (Blanco *et al.,* 1994).

Streptomyces coelicolor is the genetically best studied *Streptomyces* species. The fact that this organism produces at least five secondary metabolites has been exploited to study its developmental process genetically. The two pigmented antibiotics, the red tripyrolle undecylprodigiosin (Red) and the blue polyketide actinorhodin (Act), as well as the spore associated grey polyketide pigment (Grey) allowed, indeed, the isolation of genetic determinants controlling physiological and/or morphological differentiation of *S. coelicolor.*

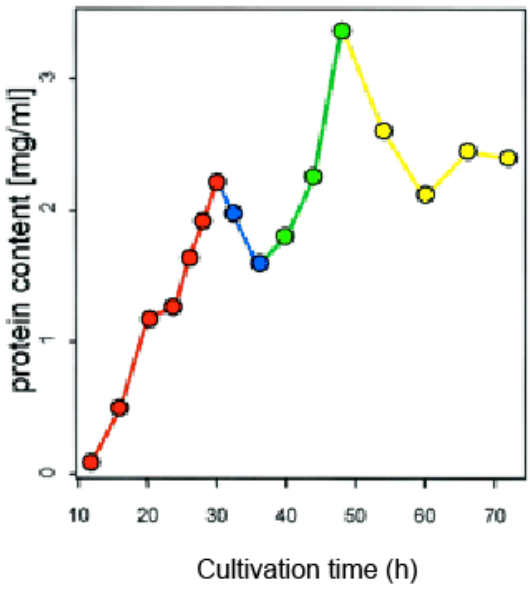

Fig. 2. Developmental phases of *S. coelicolor* J1501 cultured in a defined liquid minimal medium. Red indicates the rapid growth phase 1 (RG1); blue indicates transition phase (T-phase); green corresponds to the rapid growth phase II (RG2) and yellow to stationary phase (S-phase) (taken from Vohradsky *et al.,* 2000).

Regulation of Antibiotics Biosynthesis

Streptomycetes are notable for their ability to produce a wide variety of pharmaceutically useful compounds as secondary metabolites. These include anti-viral, anti-cancer, modulators of immune response, herbicides, insecticides, anti-parasitic compounds and nearly two-thirds of currently available antibiotics (Thompson *et al.,* 2002). They are synthesized by two families of multifunctional and multimodular enzymes, the polyketide syntheses (PKS) and the non-ribosomal peptide synthases (NRPS) through condensation reactions that assemble acyl-CoA or amino acid derivatives into polyketide and polypeptide structures. Like most *Streptomycetes*, *Streptomyces coelicolor* produces diverse antibiotics: the red-pigmented tripyrrole undecylprodigiosin (Red), the lipopeptide calcium-dependent antibiotic (CDA), and the deep blue-pigmented actinorhodin (Act) (Hopwood *et al.,* 1995). Completion of the genome sequence of *S. coelicolor* led to the prediction of about two dozen for secondary metabolites, including siderophores, hopanoids (sterol-like bacterial lipids), butyrolactones (bacterial pheromones), terpene compounds, and others (Bentley *et al.,* 2002). The genes of a secondary metabolic pathway are typically coregulated and clustered (about 20-25 kb) contiguously on the chromosome (Hopwood *et al.,* 1995) in secondary metabolic islands (SMILES) (Thopmson *et al.,* 2002). Like pathogenicity islands, SMILEs were likely acquired by horizontal transmission as suggested by the observation that genes for illegitimate recombination, such as those encoding integrases and transposases, are occasionally identified within or adjacent to these gene clusters. In addition to genes needed for the biosynthesis of secondary metabolites (antibiotics), these clusters encode also enzymes required for antibiotic resistance and pathway specific transcriptional regulators, SARPs, which activate directly transcription of the biosynthetic genes of the cluster (Hopwood *et al.,* 1995; Bibb, 1996). In *S. coelicolor*, such genes include *actII-orf4* for actinorhodin biosynthesis (Fernandez-Moreno *et al.,* 1991; Arias *et al.,* 1999), *redD* and *redZ* for undecylprodigiosin biosynthesis (Takano *et al.,* 1992; White and Bibb, 1997) and *cdaR* for production of the calcium-dependent antibiotic (Ryding *et al.,* 2002). Earlier studies showed that antibiotic production is activated when, in response to specific environmental and physiological signals, transcription of the pathway specific transcription activators have reached a threshold in the cells. In addition to this regulatory level, mediated by SARPs, “higher level” regulatory genes largely situated outside of biosynthetic gene clusters exert pleiotropic effects on production of multiple secondary metabolites, or on secondary metabolism and morphological differentiation (e.g. *cya*,

citA, *bld* genes) (Huang *et al.,* 2005; Viollier *et al.,* 2001; Süsstrunk *et al.,* 1998). These genes are involved in regulatory events needed to perceive metabolic imbalances that interrupt or arrest growth. Antibiotic biosynthesis is, in fact, activated in a growth-phase dependent manner, coincident with the onset of aerial hyphae formation in agar-grown cultures and stationary phase in liquid (Hopwood *et al.,* 1995). Stresses generated by nutritional limitations and cell density can lead to the accumulation of secondary messangers such as cAMP, ppGpp and γ-butyrolactones, which activate the secondary metabolism. A role for cAMP in antibiotic biosynthesis was suggested by the observation that a disrupted mutant in the *S. coelicolor cya* gene, encoding adenylate cyclase, was defective in actinorhodin biosynthesis. Moreover, the fact that the synthesis of the blue pigmented antibiotic could be induced by concentrations of cAMP (< 20 μM) found in the spent medium of *S. coelicolor* cultures, suggested that it may serve as diffusible signaling molecule to switch from primary to secondary metabolism (Süsstrunk *et al.,* 1998; see also below). Antibiotic biosynthesis has also been associated with the intracellular accumulation of the stringent factor ppGpp (Hesketh *et al.,* 2001; Ochi, 1987). In *S. coelicolor* accumulation of ppGpp occurs transiently in response to amino acid limitation (stringent response) and is developmentally regulated (T-phase and stationary phase) (Chakraburtty *et al.,* 1996; Ochi, 1990). Its synthesis requires the products of both *relA* and *relC* genes. In particular, when mutations are introduced in ether *relA* (Chakraburtty and Bibb, 1997) or *relC* (Ochi, 1990), *S. coelicolor* mutants are not only defective in ppGpp biosynthesis but also in production of Act and Red under conditions of nitrogen limitation, suggesting a direct role for ppGpp in transcriptional activation of the pathway specific activators, *actII-orf4* and *redD* (Hesketh *et al.,* 2001).

Other signals, playing important roles in the onset of antibiotic production are generated by the *Streptomyces* quorum sensing mediators, γ-butyrolactones (Horinuchi and Beppu, 1994). The first and best studied γ-butyrolactone, required for both streptomycin production and sporulation in *Streptomyces griseus*, is A-factor (for a review Horinouchi, 2002). This is synthesized by the product of the *afsA* gene (Horinouchi *et al.,* 1989) and accumulates in the culture medium, before the onset of streptomycin biosynthesis. It diffuses freely across the cytoplasmic membrane and it binds with high affinity to a cytoplasmic A-factor binding protein, ArpA (Onaka *et al.,* 1995). Upon A-factor binding, ArpA releases the transcriptional repression of the pleiotropic regulatory gene, *adpA*, encoding an AraC-like transcription factor which directly activates transcription of genes involved in secondary metabolism (e.g.

strR for streptomycin biosynthesis and resistence) and morphological differentiation (e.g *amfR*; see below) (Ohnishi *et al.,* 1999; 2005).

Similar, but more complex regulatory cascades involving γ-butyrolactones and cognate ArpA-like binding proteins have been reported in *S. virginiae* (*barAB*; Okamoto *et al.,* 1995) for virginiamycin production; in *S. fradiae* (*tylPQTS*; Wietzorrek and Bibb, 1997) for tylosin biosynthesis; in *S. pristinaespiralis* (*spbR* and *papR1*; Folcher *et al.,* 2001) for pristinamycin biosynthesis and in *S. coelicolor* (SCB1, *scbA* and *scbR*; Takano *et al.,* 2001) for Act and Red production.

Screening of antibiotic defective mutants in *Streptomyces coelicolor* and *Stretomyces lividans* (Act^+, Red^+), have led to the identification of additional regulatory genes, encoding members of two component transduction systems (TCS; see below). In particular, disruption of *cutRS* (in *S. lividans*) or *absA1-2* (in *S. coelicolor*), resulted in overproduction of actinorhodin on both solid and liquid cultures, while their overexpression in *S. coelicolor* repressed antibiotic biosynthesis (Hutchings *et al.,* 2004).

Another checkpoint for antibiotic biosynthesis is exerted by the pleiotropic *bld* mutants of *S. coelicolor*. In fact, most of *bld* mutants (e.g. *bldA*, *bldC*, *bldG*, *bldH*, *bldJ*, *brgA*, *citA* and others; see below), in addition to their incapability to erect aerial hyphae, are not able to synthesize pigmented antibiotics (Act^- and/or Red^-). In the case of *bldA*, the effect on production of Act and Red is largely understood. Isolation of *bldA* gene identified as the only tRNA in *S. coelicolor* that can efficiently translate the rare leucine codon UUA (Leskiw *et al.,* 1991). This implied that the regulatory role of *bldA* on antibiotic production was likely to be posttranscriptional. Indeed, *actII-orf4* and *redZ* (encoding a response-regulator-like protein that directly activates the expression of the undecylprodigiosin specific transcription activator, *redD*) contain a UUA codon, which requires the tRNA product of *bldA* gene to be efficiently translated. This was demonstrated by the observation that changes of the UUA codon into the more abundant leucine CUC codon in *actII-orf4* resulted in actinorhodin production in the *bldA* mutant (Fernandez-Moreno *et al.,* 1991).

Recent work, based on microarray analyses (Huang *et al.,* 2005) has demonstrated, furthermore, that antibiotic biosynthesis is regulated by an even more complicated network of cross-regulation between pathway specific regulators. SARPs, in fact, originally thought to regulate only the genes of their own gene cluster, can also control other antibiotic biosynthetic pathways and exert effects on the expression of previously characterized pleiotropic “high level regulators”.

Aerial Hyphae Formation (AHF)

The unique developmental characteristics of members of the genus *Streptomyces* have generated considerable interest in their genetics and physiology. *Streptomyces* mutants that fail to develop aerial hyphae are called *bld* (bald) mutants. Those, instead, that produce aerial hyphae that do not generate normal mature spores are called *whi* (white) mutants (Fig 3; see below).

Most of *bld* mutants, until now identified, have been isolated from *Streptomyces coelicolor*, the genetically best studied *Streptomyces* species. Although dozens of *bld* mutants have been already isolated about 20 years ago, only few *bld* genes have been studied at molecular levels. Most of the known *bld* genes have turned out to encode regulatory factors, exerting pleiotropic effects on both morphological and physiological differentiation. Indeed, in addition to their morphological block, many *bld* mutants (*bldA*, *bldB*, *bldC*, *bldG*, *bldH*, others) of *S. coelicolor* are also unable to make pigmented antibiotics such as actinorhodin and undecylprodigiosin.

bldA encodes the only tRNA species able to read the leucine codon UUA efficiently, implying the involvement of a TTA-containing gene in initiating aerial growth (Takano *et al.,* 2003; Nguyen *et al.,* 2003). Studies of intracellular complementation have demonstrated that such a gene, needed for morphological differentiation corresponded to the pleiotropic *S. coelicolor adpAc* gene (see below). Unlike the TTA-version, engineered forms of *adpAc*, where the TTA codon was replaced by the TTG or CTC leucine codones by site-directed mutagenesis, were able to restore aerial hyphae formation (AHF) when expressed in the *bldA* mutant. This indicated that the UUA codon of the *adpAc* mRNA is the principal target through which *bldA* influences morphological differentiation. Moreover the observation that *adpA(TTA)* could complement the *bldH* mutation and that the *adpAc* gene cloned from the *bldH* mutant contained an additional G in its coding sequence confirmed that *adpAc* is a frame shift mutation of *bldH* (Takano *et al.,* 2003; Nguyen *et al.,* 2003).

In addition to *bldA* and *bldH*, other *bld* loci have been characterized at molecular level. *bldB* (Pope *et al.,* 1998), *bldD* (Elliot *et al.,* 1998) and *bldC* (Hunt *et al.,* 2005) encode small DNA-binding proteins while *bldG* codes for an anti-anti-sigma factor (Bignell *et al.,* 2000) homologous to the *Bacillus subtilis* anti-anti-sigma factor, RsbV, regulating the activity of the stress response sigma factor, σ^B. Although several *bld* loci encode products that are associated directly with gene expression, the product of the *bldJ* gene

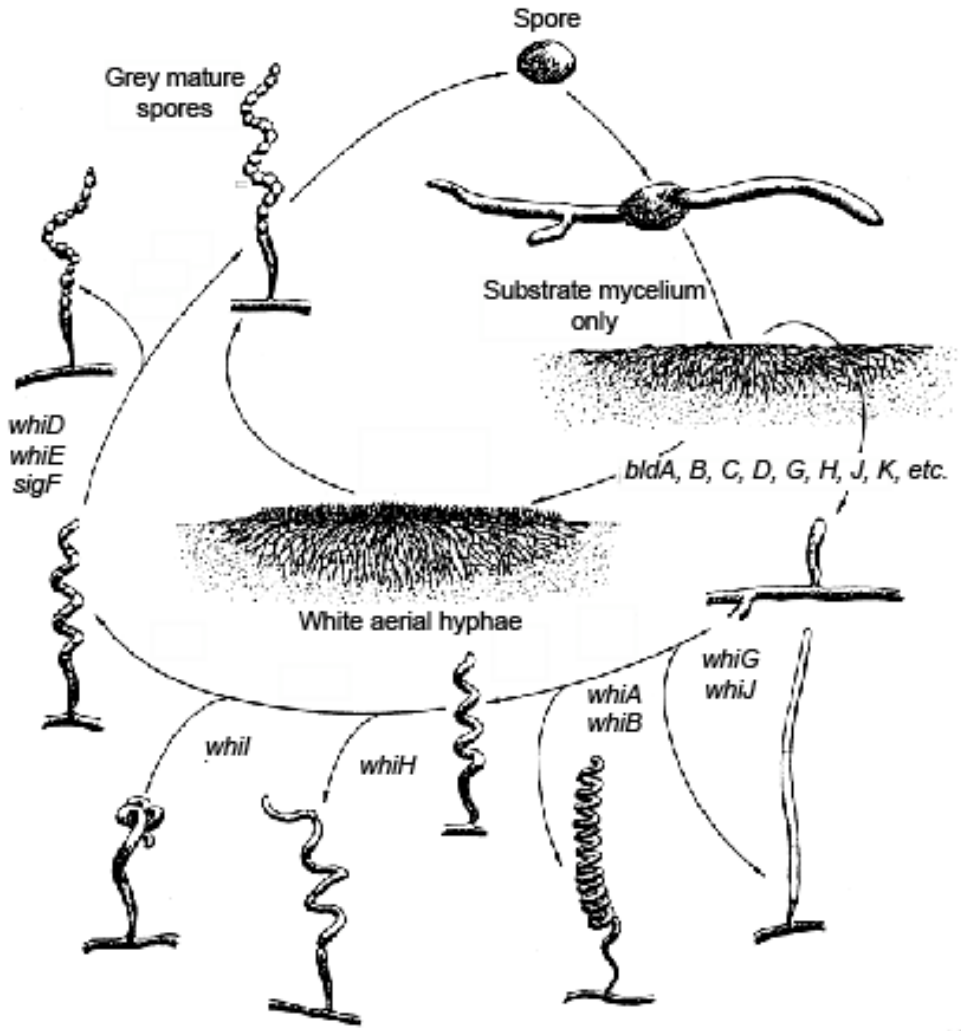

Fig. 3. Roles of developmental genes in the morphological differentiation program of *S. coelicolor* (Taken from Chater, 1998).

(Willey *et al.,* 1993) is a small secreted peptide that is imported inside the hyphal cells by the BldK oligopeptide permease complex, as predicted by studies of extracellular complementation (see below; Nodwell *et al.,* 1996). *bldK* locus consists of five genes that specify homologs of ABC transporters. Because *bldK* mutations confer resistance to the toxic tripeptide bialaphos, it was inferred that BldK is an oligopeptide importer.

The morphological defect exhibited by *bld* mutants is in part due to their incapability to trigger the biosynthesis of a morphogenetic biosurfactant compound, SapB (see below), when grown on complex agar medium (Willey *et al.,* 1991; 1993). Indeed, extracellular addition of purified SapB restored to *bld* mutants their capacity to erect aerial hyphae, albeit without subsequent spore formation (Tillotson *et al.,* 1998).

Other signals regulating morphological differentiation in *Streptomycetes* are the microbial pheromones, γ-butyrolactones. A-factor of *S. griseus* is the most intensively and extensively studied γ-butyrolactone (for a review see Ohnishi *et al.,* 2005). A-factor accumulates in the medium during growth and reaches a critical concentration before the onset of AHF and streptomycin biosynthesis. It diffuses freely across the cytoplasmic membrane into the cells where it binds to the A-factor receptor protein, ArpA. Upon binding, A-factor dissociates the DNA-bound ArpA from the DNA, driving transcription of the pliotropic regulator gene, *adpA*. AdpA, in turn, directly activates transcription of morphogenetic genes, such as *amfR* (see below) and *adsA* (encoding an ECF sigma factor). Although *S. coelicolor* orthologues of *arpA* (*cprA* and *cprB*; Onaka *et al.,* 1998), *adpA(bldH)* (Takano *et al.,* 2003; Nguyen *et al.,* 2003), *amfR* (*ramR* in *S. coelicolor*; Ma and Kendall, 1994; Keijser *et al.,* 2002; Nguyen *et al.,* 2002; O'Connor *et al.,* 2002), and *adsN* (*bldN* in S. coelicolor; Bibb *et al.,* 2000) were shown to play similar roles in antibiotic production and/or in morphological differentiation, different regulatory systems control the expression of these genes in *S. coelicolor* throughout development. *S. coelicolor adpA*, for example, is not under control of γ-butyrolactones (SCB1; Takano *et al.,* 2003) and *bldN* (*adsA* orthologue) is not activated by AdpA, but it is under developmental control by BldD (Bibb *et al.,* 2000).

Stress and Development

Several *bld* mutants can undergo normal development, producing sporulating aerial hyphae, on minimal media using non acidogenic carbon sources such as mannitol. The conditional *bld* phenotype, exhibited by several *bld* mutants, suggested that development in *Streptomyces* has to proceed through various nutritional, stress and physiological checkpoints. The first evidence that developmental switches are intimately connected to the nutritional state of the colony was provided by the observation that guanosine nucleotides were involved in the activation of the developmental program (Ochi, 1987). In particular, while production of the stringent response factor, ppGpp was associated to the activation of the secondary metabolism, decrease in the intracellular GTP pool was related to morphological differentiation. In addition, overexpression of *obg*, encoding a GTP-binding protein, was found to suppress aerial hyphae formation in *S. coelicolor*. This was interpreted as Obg, by monitoring the intracellular GTP pool size, may sense nutritional changes that ultimately lead to morphological differentiation (Okamoto and Ochi, 1998). Similar phenomena associated with the activation of the sporulation program were described in *Bacillus subtilis* (Freese and Heinze, 1984).

The hypothesis that *bld* mutants were unable to initiate morphological differentiation because incapable to sense and/or signal switches in primary metabolism, was reinforced by studies of catabolite repression (Pope *et al.,* 1996). It was found, indeed, that all of the *bld* mutants tested, were defective in the regulation of the glucose-sensitive and galactose-dependent *galP1* promoter that directs expression of the galactose utilization operon. One mutant, *bldB*, was analyzed further and shown also to be catabolite derepressed for genes for glycerol utilization and agar decomposition. The importance of the switching to an alternative metabolism for the initiation of the developmental program in *S. coelicolor* was further supported by studies of the conditional *bld* mutants, *cya*, *citA* and *acoA* (Süsstrunk *et al.,* 1998; Viollier *et al.*, 2001a; 2001b). *cya* encodes the *S. coelicolor* adenylate cyclase for biosynthesis of cAMP. In addition to a severe delay in spore germination, a disrupted *cya* mutant, grown on solid glucose minimal medium, exhibited also a block in AHF. Such developmental arrest was brought about by excretion of organic acids produced during the first rapid growth phase, which generated vegetative mycelium. Unlike the wild type (*cya*+) strain, in fact, *cya* mutant was not able to neutralize its medium, probably by reincorporating and metabolizing extracellular acids. These defects could be suppressed by high concentration of cAMP or during growth on buffered medium, suggesting a role for cAMP in signaling neutralization of the medium and subsequent activation of the developmental program

(Süsstrunk *et al.,* 1998). Analogously, mutants in *citA*, encoding the TCA citrate synthase, and in *acoA*, encoding the TCA aconitase, accumulated organic acids in unbuffered glucose containing medium, exhibiting defects in AHF and in the synthesis of pigmented antibiotics (Viollier *et al.*, 2001a; 2001b). Interestingly also other *bld* mutants (*bldA*, *bldB*, *bldC*, *bldD, bldG*) irreversibly acidify glucose containing media. This suggests that the accumulation of acidic toxic organic compounds, generated by imbalances in the flux of the TCA cycle, may arrest growth and the initiation of the developmental program.

All these studies demonstrated that developmental changes in *Streptomyces* are triggered by nutritional limitations. However as depolymerization of storage compounds (glycogen and trehalose) is part of the developmental program to provide carbon sources and turgor pressure for AHF and sporulation (Chater, 1998), it was inferred that such osmotic and nutritional changes presumably activated adaptive stress response systems during development. In unicellular bacteria, such as *Bacillus subtilis*, diverse stresses (heat shock, cold shock, slat and ethanol stresses) induce a common set of proteins, called general stress proteins (GSPs; Hecker *et al.,* 1996). In contrast, proteomic studies showed that in *Streptomyces coelicolor* various stresses induced independent sets of proteins or stimulons (Vohradsky *et al.,* 2000). Importantly, many cold, heat and osmotic shock proteins were also developmentally regulated, being coordinately induced or repressed during the T-phase (Bucca *et al.,* 1995; Puglia *et al.,* 1995; Vohradsky *et al.,* 2000). Thus stress regulatory systems controlling these stimulons are integral parts of the developmental program in *S. coelicolor*.

In *Bacillus subtilis* the general stress response is controlled by σ^B (*sigB*), whose activity is regulated by phosphorilation and protein-protein interactions with the anti-sigma factor, RsbW, and the anti-anti-sigma factor, RsbV. Genome sequence analysis (Bentley *et al.,* 2002) has revealed that *S. coelicolor* encodes at least nine *sigB* homologues as well as numerous anti-sigma factors (RsbW orthologues) and anti-anti-sigma factors (RsbV orthologues), likely reflecting a higher complexity in the stress response regulation.

A direct and intimate connection between stress and development in *S. coelicolor* has been provided by studies of the *B. subtilis sigB* homologues *sigH*, *sigB* and *sigF* (Viollier *et al.,* 2003a; Viollier *et al.,* 2003a; Kelemen *et al.,* 2001; Lee *et al.,* 2005; Potuckova *et al.,* 1995). In the case of σ^H, deletion of the *sigH* structural gene has no obvious morphological effect, but simultaneous deletion of the cotranscribed *prsH* gene, encoding an anti-σ^H factor, produces a *bld* phenotype. This indicated that interaction of PrsH with another target mediates a developmental checkpoint (Viollier *et al.,* 2003a). In addition of being

induced by heat or ethanol stresses, *sigH* expression is developmentally controlled (Viollier *et al.,* 2003a; Kelemen *et al.,* 2001). While *sigHp1* activity is confined to rapid growth phase 1 or vegetative mycelium, transcription of *sigHp2* is dramatically induced at the onset of AHF and is spatially restricted to sporulating aerial hyphae. Such temporal and spatial regulation of *sigHp2* activity during development is mediated directly by the repressor protein BldD, suggesting that premature and deregulated expression of *sigH* might be in part responsible of the developmental arrest in the *bldD* mutant. Moreover σ^H undergoes a developmentally regulated post-transcriptional control, as its SigH-$\sigma^{51/52}$ isoform is mainly expressed in the vegetative mycelium, while its SigH-σ^{37} product is maximally expressed during AHF (Viollier *et al.*, 2003b). Roles in osmotic stress response and development have been also described for other *B. subtilis* σ^B orthologues, such as σ^B, σ^L and σ^M in AHF (Lee *et al.,* 2005), and σ^F in sporulation (Potuckova *et al.,* 1995).

Extracellular Signaling Cascade (ESC)

The observation that extracellular factors are involved in the differentiation process of *Streptomyces*, was first described in *S. griseus*. Purification, structural determination and chemical synthesis of this compound, defined the γ-butyrolactone, A-Factor as the first pheromone-like molecule described in bacteria (Horinouchi and Beppu, 1992; 1994). In this case the developmental block exhibited by a mutant strain defective in A-factor biosynthesis was restored by growth of the wild type strain in close proximity. This was interpreted as diffusion of A-Factor from the wild type allowed the mutant to overcome the developmental block. Analogous extracellular complementation experiments have demonstrated the existence of multiple extracellular signals governing the fruiting body formation by the social bacterium *Myxococcus xanthus* (Kaiser 1989).

Previous studies have shown that extracellular complementation governs morphological differentiation also in *Streptomyces coelicolor*. When certain pairs of *bld* mutants are grown in close proximity, one *bld* mutant may function as the donor of an extracellular signal, which triggers AHF in colonies of a nearby recipient *bld* strain. Since this effect was partner dependent, it was possible to define a hierarchical classification of these mutants. In the simplest model, each *bld* gene is involved directly or indirectly in the synthesis, perception of or response to one of six several extracellular signaling molecules in the cascade. This model posits that each signal induces the production of the next one, ultimately leading to the synthesis of the morphogenetic compound SapB (see below), needed for the emergence of aerial hyphae (Fig 4; Willey *et al.,* 1991; 1993; Nodwell *et al.,* 1999; Nguyen *et al.,* 2002). Thus the *bldJ* mutant, which can be stimulated by all the other mutants that define the cascade, but cannot itself stimulate any of them, is presumably defective in the first signal, signal 1. Nodwell *et al.* (1996) proposed that signal 1 is an extracellular oligopeptide, which is uptaken by the BldK oligopeptide permease. This leads, via unknown genes, to production of signal 2. Since *bldA* and *bldH* mutants stimulate *bldJ* and *bldK* mutants, but no others, they must be defective in the recognition or uptake of signal 2, or in the production of signal 3. Similar logic must implicates *bldG* at some stage between signals 3 and 4, *bldC* between signals 4 and 5, and *bldD* between signal 5 and the production of SapB (Fig 4). However, an alternative to this model is that extracellular complementation of a recipient *bld* mutant by the wild type strain or another donor *bld* mutant may be the result of the inactivation or detoxification of the medium from excreted toxic compounds, such as organic acids (Süsstrunk *et al.,* 1998; Viollier *et al.,* 2001).

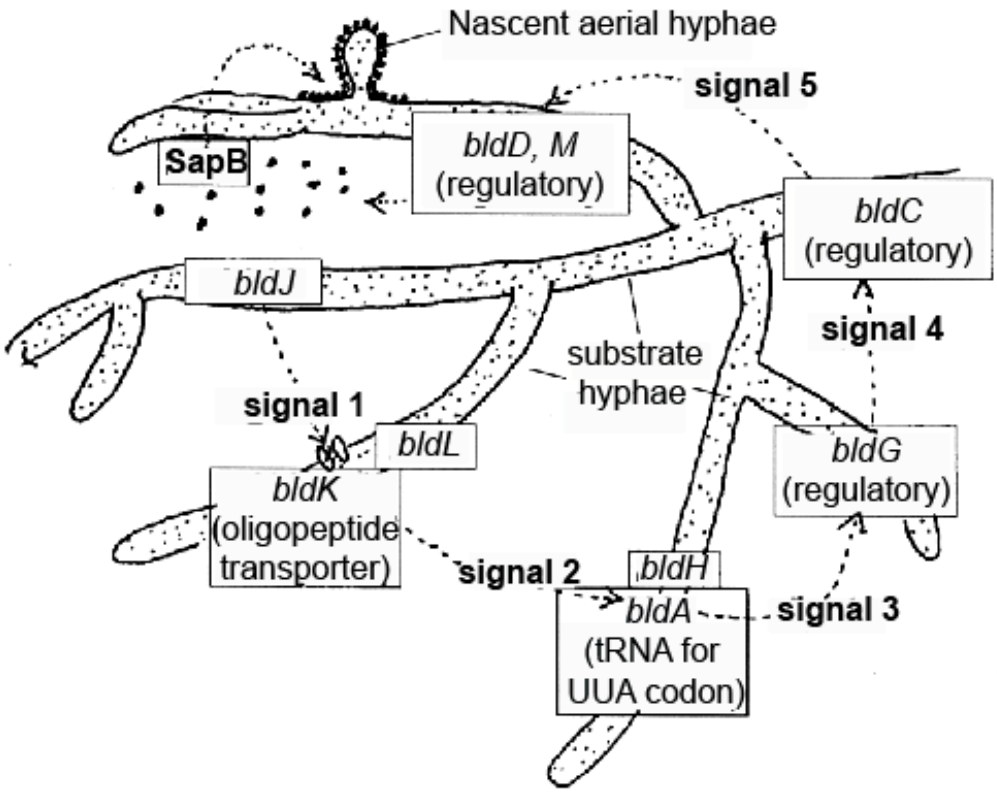

Fig. 4. *bld* genes of *S. coelicolor* and their roles in extracellular signalling. The scheme is based on the pattern of interactions among different *bld* mutants, some of which exhibit the ability to cause restoration of aerial growth to others (Taken from Chater, 1998).

This scenario does not exclude the possibility that positively acting molecules are also involved. For example, if we assume that the developmental block, exhibited by a *bld* recipient strain is due to the accumulation of a growth inhibitory substance, it is conceivable that the *bld* donor strain secretes a positively acting factor(s) causing a metabolic switch. This, in turn, might result in detoxification and thus resumption of growth and differentiation.

The principle of the signaling cascade relies on a fundamental concept in biology. Important cellular events, such as morphological and physiological differentiation, are only initiated after the current physiological status of the cell has been assessed and all of the appropriate requirements have been met (cellular checkpoints). Thus, the signaling cascade might represent such a checkpoint at which physiological inputs are integrated for the activation of the developmental program.

Amphipatic Compounds, SapB and the *ram* gene cluster

To develop aerial hyphae, filamentous microorganisms must form hydrophobic filaments that can break surface tension and project into the air, where they differentiate into spores or fruiting bodies. Both filamentous fungi and bacteria have independently evolved the ability to form aerial structures. Recent investigations in *Streptomyces coelicolor* have demonstrated that remarkable parallels exist in the molecular biology of aerial development in fungi and bacteria (for a review see Talbot, 2003).

Filamentous fungi grow into the air with the aid of surface active low molecular weight proteins called hydrophobins, produced by diverse members of *Ascomycetes* and *Basidiomycetes* (Wösten, 2001). In filamentous bacteria, the first morphogenetic compound found to be involved in AHF was the *s*pore-*a*ssociated-*p*rotein, SapB. This molecule is produced and secreted during growth conditions on rich media and appears to be important for morphogenesis under these conditions. The most compelling evidence for this is the observation that application of purified SapB to *bld* mutants restores their ability to form aerial filaments (Willey *et al.,* 1991). Consistent with this, SapB biosynthesis was found to be dependent on all *bld* genes and to be spatially confined to aerial hyphae and spores (Willey *et al.,* 1991). Moreover, these experiments showed also that SapB-rescued *bld* mutants formed aerial filaments that did not go on to sporulation; rather they resembled short branching vegetative hyphae that were simply released from the colony surface to stand erect. This was consistent with a primarily role for SapB as biosurfactant. Similar to hydrophobins (Wösten, 2001), SapB self-assembles at the air-colony interface through hydrophobic interactions where it reduces the surface tension and enables the upward growth of aerial hyphae (Fig. 5). The functional similarity between SapB and fungal hydrophobins was further demonstrated by the capacity of the SC3 hydrophobin from *Schizophyllum commune* to rescue AHF in *S. coelicolor bld* mutants (Tillotson *et al.,* 1998). Interestingly, SapB and SC3 do not share any common structural feature (see below), but rather a similar biosurfactant activity needed for AHF. Nevertheless, there is some specificity to surfactant action: application of the *Bacillus subtilis* surfactants surfactin and fengicin, and the *Pseudomonas aeruginosa* product viscosin has no effect on *bld* mutants (Richter *et al.,* 1998).

In a recent work, Kodani *et al.* (2004) have resolved the structure of SapB and found that SapB is a lantibiotic-like molecule derived from the 42 amino acid product of *ramS*, a member of the *ram* gene cluster. The *ram* gene cluster in *S. coelicolor* is orthologous to the *S. griseus amf* gene cluster, and was

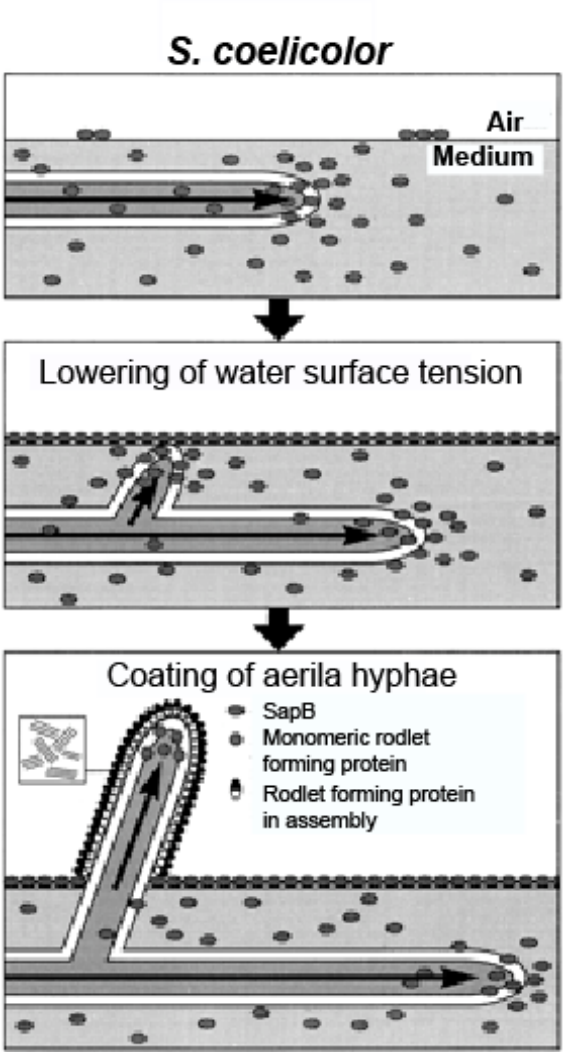

Fig. 5. Model for the action of surface-active proteins in aerial hyphae formation in bacteria (modified from Talbot, 2003).

found to be highly conserved in several *Streptomyces* species (Fig. 6A). Overexpression of the *amf* genes in *S. griseus* restored AHF to a developmentally blocked mutant deficient in A-Factor (Ueda *et al.,* 1993). Similarly, in *S. coelicolor* the *ram* genes stimulated rapid formation of aerial hyphae when present at high copy number (Ma and Kendal, 1994). The *ram* (*amf*) gene cluster consists of the four genes of the *ramCSAB* (*amfTSAB*) operon (Nguyen *et al.,* 2002; Keijser *et al.,* 2002), and on the opposite strand, of the monocistronic gene *ramR* (amfR; see below) (Fig 6A). Conversion of RamS peptide into SapB is characterized by extensive posttranslational modifications, during which dehydration of specific serine residues give rise to highly reactive didehydroalanine (Dha) derivatives. These react with the sulphydral groups of cysteine residues to form acid-stable thioether cross-links, referred to as lanthionine (Lan) bridges. Finally the peptide is exported and a specific peptidase cleaves the leader peptide (corresponding to the first 21 amino acids in RamS) to release the mature SapB (Fig. 6B). The Lan-bridging pattern yields two hydrophobic cyclic structures, which confer an overall amphiphilicity required for biosurfactant activity (Kodani *et al.,* 2004). A significant difference, however, between SapB and the lantibiotics is that SapB appears to lack antimicrobial activity (Kodani *et al.,* 2004). Consistent with the origin of SapB from RamS, a *ramS* insertion mutant is severely delayed in AHF on complex medium (Nguyen *et al.,* 2002). Genetic studies as well as analyses based on protein sequence homologies have predicted a role(s) for the other members of the *ramCSAB* operon in SapB biosynthesis. In particular, although there are conflicting reports about the importance of RamA and RamB in development (Ma and Kendal, 1994; Nguyen *et al.,* 2002; Ueda *et al.,* 2002) it was proposed that RamA and RamB, encoding subunits of an ABC transporter, may provide the exporter for SapB. So far, the strongest evidence that RamC (904 amino acid residues) is the likely RamS modifying enzyme is the sequence similarity between its C-terminal domain with the LanM-type dehydratases, CinM and MrsM (Kodani et al., 2004), required for the synthesis of the lantibiotic compounds, mersacidin and cinnemycin (Widdick *et al.,* 2003; Altena *et al.,* 2000). Less obvious is, instead, the role of the N-terminal domain of RamC. This contains sequence motifs related to those found in Ser/Thr kinases and a domain required for homodimarization and association to the plasma membrane (Hudson *et al.,* 2002; Hudson and Nodwell, 2004). The importance of *ramC* in development was confirmed by genetic studies. In fact, similarly to *bld* mutants, the *ramC* mutant is developmentally blocked in AHF, but is not pleiotropically defective in antibiotic biosynthesis (Nguyen *et al.,* 2002; O'Connor *et al.,* 2002; Hudson *et al.,* 2002).

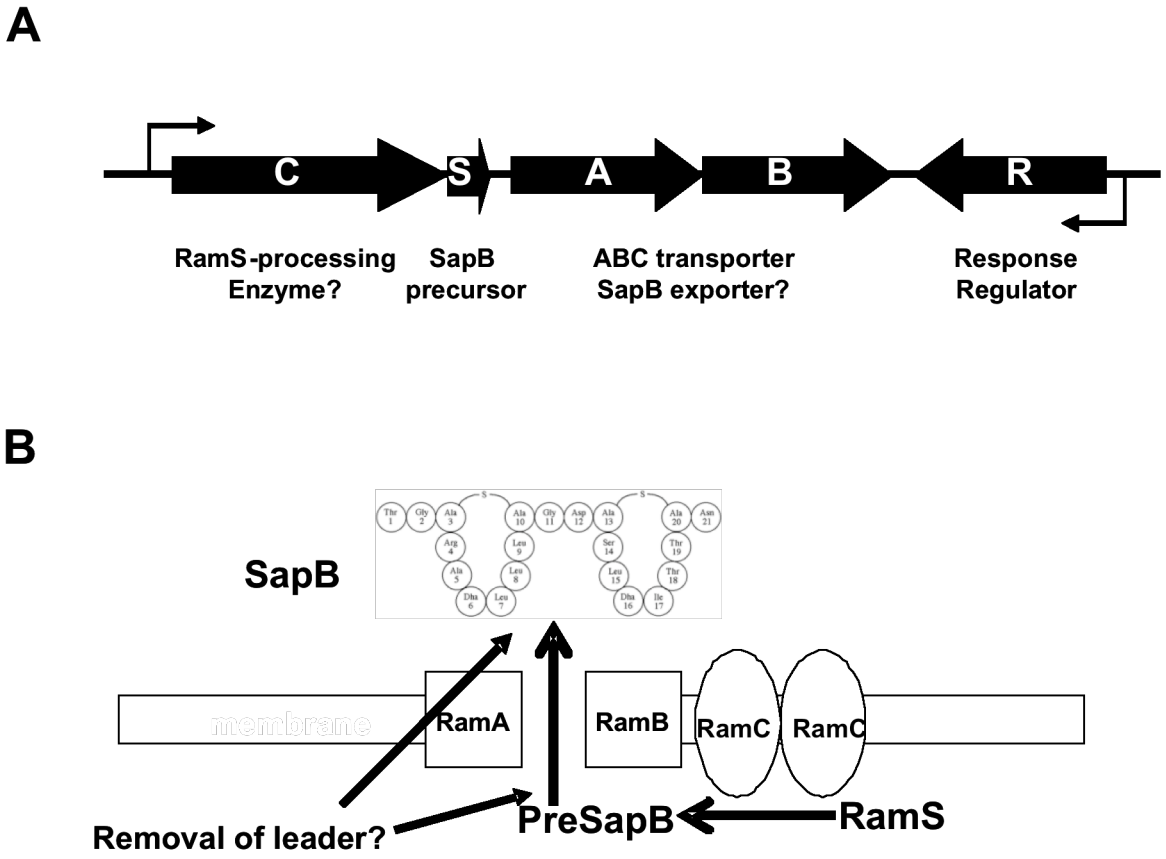

Fig. 6. Model for SapB maturation.
(A) The *ram* gene cluster.
(B) The *ramS* gene product is modified by dehydration and thioether formation, presumably catalysed by RamC that is known to function as a dimer and is associated to the membrane. The modified product, PreSapB is exported and the leader sequence is cleaved to yield mature SapB. It is unclear if processing occurs before, during or following export (modified from Willey *et al.,* 2005).

In addition to SapB, another lanthionine(Lan)-containing peptide SapT was recently isolated from *S. tendae*. Similar to SapB, SapT can drive aerial hyphae formation of *S. coelicolor bld* mutants (Kodani *et al.,* 2005). Although SapT is functionally similar to SapB and is a Lan-containing peptide, its amphipatic structure differs markedly from SapB. Nevertheless, these molecules that exhibit cross-species function have conserved the key amphiphilicity that allows them to function as biosurfactants (Kodani *et al.,* 2005).

The dispensability of SapB for AHF during development on minimal medium showed clearly that alternative pathways to make aerial filaments must exist in *S. coelicolor* (Willey *et al.,* 1993). This led to further investigations and the isolation of the rodlin proteins (Claessen *et al.,* 2002). These are encoded by the homologous genes, *rdlA* and *rdlB*, which, similarly to fungal hydrophobins, are required for formation of the rodlet layer. This is made up of a mosaic of 8- to 10-nm-wide parallel rods, intimately associated to aerial hyphae and spores. Rodlins are necessary, however, for adhesion to hydrophobic surfaces but not for erection of aerial hyphae (Claessen *et al.,* 2002; 2004).

Subsequent discovery of the "*c*oelicolor *h*ydrophobic *a*erial *p*roteins", chaplins, showed that this group of proteins is essential for AHF on several media (Claessen *et al.,* 2003; 2004; Elliot *et al.,* 2003). Chaplins is a family of eight highly conserved amphiphilic proteins, sharing a region of similarity (the chaplin domain) characterized by a very high hydrophobic amino acid content (60-65%). Three of the chaplins, ChpA-C, are proteins of 210-230 residues and contain two chaplin domains. The second group of five chaplins, ChpD-H, are proteins of 50-63 amino acids and contain one single chaplin domain. Structural predictions indicate that chaplins are rich in hydrophobic β-sheets, which confer them the ability to aggregate into a surface active amyloid-like film (Claessen *et al.,* 2003). Interestingly, the long chaplins, ChpA-C appear to be substrates for the cell wall transpeptidase enzyme sortase, which is involved in incorporating proteins into the peptidoglycan layers (Elliot *et al.,* 2003). This suggested that ChpA-C may facilitate attachment of the shorter chaplins to the surface of emerging aerial hyphae, helping them to breach the air-water interface and escape from the embedded vegetative mycelium. *chp* genes (*chpABCDEFGH*) are transcribed at the onset of AHF and are developmentally regulated by *bld* genes (Claessen *et al.,* 2004; Elliot *et al.,* 2003). Their requirement for AHF was demonstrated by the progressive deletion of *chp* genes (Claessen *et al.,* 2003; 2004; Elliot *et al.,* 2003). Strains lacking up to six chaplins were severely delayed in AHF, while deletion of all eight chaplin genes led to a *bld* phenotype.

In addition to chaplins and SapB, *S. coelicolor* genome (Bentley *et al.,* 2002) has the potentiality to encode several other secreted hydrophobic proteins, whose roles in AHF await to be defined.

Spore Maturation

The fate of aerial hyphae is to produce spores. Mutants of *S. coelicolor* that are defective in sporulation have been isolated by virtue of their white colour (*whi*). These mutants, indeed, fail to make the polyketide grey pigment normally associated with spore biogenesis. Genetic epistasis experiments have defined "early" *whi* loci which act in the following hierarchical order: *whiG, whiJ>whiA, whiB>whiH>whiI* (Fig 3; Fig 7; Chater 1993). These genes are needed for commitment of aerial filaments to the typical aerial hyphae mode of growth and to compartmentalization into unigenomic prespore compartments. Furthermore early *whi* genes activate expression of the "late" *whi* genes involved in cell wall spore formation and spore maturation (Chater, 1998).

A crucial early *whi* gene is *whiG* (Chater, 2001). A *whiG* mutant exhibits characteristic straight aerial hyphae, which have normally spaced vegetative-like septa and rare branches. When overexpressed, *whiG* leads to premature and ectopic sporulation in the vegetative mycelium, strongly suggesting that WhiG exerts a key regulatory role in the initiation of the sporulation program in *S. coelicolor* (Flärdh *et al.,* 1999). WhiG belongs to the σ^{FliA}-family of sigma factors that mediate the chemotactic response in *E. coli* and *B. subtilis* (Chater *et al.,* 1998). Since *whiG* transcription appears to be at a more or less constant level throughout development, it was argued that activity of σ^{whiG}, analogously to σ^{FliA}, is likely to be regulated by a yet unidentified anti-sigma factor (Kelemen *et al.,* 1996). Once active, σ^{whiG} RNA polymerase holoenzyme activates transcription of two further early sporulation regulatory genes, *whiH* (Ryding *et al.,* 1998) and *whiI* (Ainsa *et al.,* 2000). Both these two genes are, directly or indirectly, autoregulatory as a *whiH* mutant overexpressed *whiH* and a *whiI* mutant overexpressed *whiI*. However the sharp increase in expression of both genes around the time of sporulation septation suggests that specific signals release autorepression at this time. WhiH and WhiI belong to two different families of transcription factors (Ryding *et al.,* 1998; Ainsa *et al.,* 2000). WhiH, in particular, is a member of the GntR family of transcription regulators. These typically respond to acid carbon metabolites, which modulate their transcriptional autorepression as well as repression of genes involved in carbon metabolism, as seen in other bacteria (Ryding *et al.,* 1998). WhiI is an atypical response regulator (see below) that is unlikely phosphorylated by a cognate sensor histidine kinase as its predicted phosphorylation pocket lacks two key conserved residues needed for phosphorylation. Based on these observations and analogies, it was hypothesized that WhiH and WhI initially function as repressors of

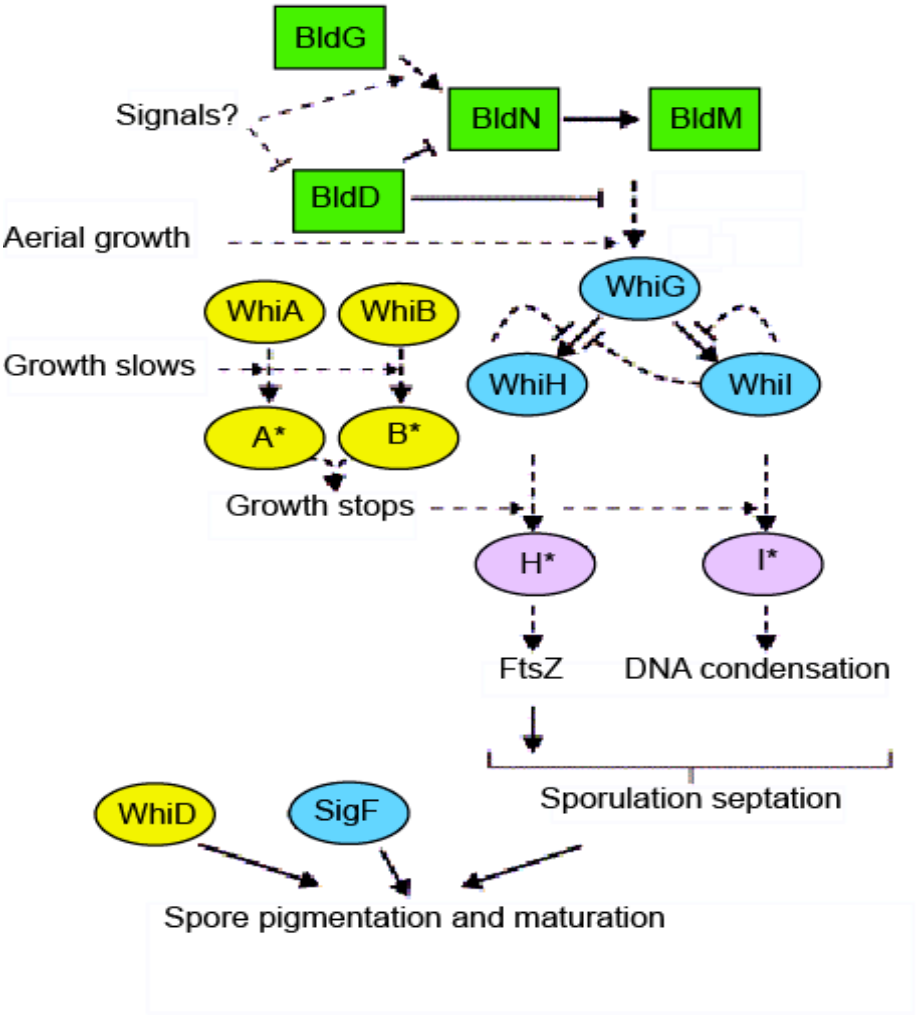

Fig. 7. Regulatory and checkpoint network for *Streptomyces coelicolor* sporulation (modified from Chater, 2001).

some target gene promoters and that signals, generated during aerial growth, cause them to adopt altered conformations (WhiH* and WhI*), in which they loose their autorepressor activities and become activators of genes involved in sporulation septation. Such a model is in agreement with the transcriptional activation dependence on *whiH* of the late sporulation genes, *ftsZ* and *sigF* (Ryding *et al.,* 1998) (Fig 7).

Other two early regulatory *whi* genes, needed for commitment to sporulation are *whiA* and *whiB* (Chater 2001; Ainsa *et al.,* 2000; Flärdh *et al.,* 1999). Each of these two genes has a low level constitutive promoter, while a second promoter is strongly transcribed at the onset of AHF. *whiA* or *whiB* mutants exhibit abnormally long and coiled aerial hyphae that are almost devoid of septation. This implyed that growth cessation may provide the basis of a *whiA*- and *whiB*-mediated developmental checkpoint for commitment to sporulation septation (Flärdh *et al.,* 1999) (Fig 3). Indeed, properly controlled aerial hyphal cell wall structure, sporulation septation, nucleoid condensation, spore pigment synthesis (*whiE*) and *sigF* transcription require *whiA* and *whi*B (Kelemen *et al.,* 1996). Clues about a possible molecular mechanism through which WhiB may regulate expression of downstream genes were provided by the biochemical characterization of the WhiB-homologue, WhiD (Jakimowicz *et al.,* 2005). Both WhiB and WhiD belong to a family of proteins only found in *Actinomycetes* (Molle *et al.,* 2000). These proteins contain four essential conserved cysteine residues, which bind a [4Fe-4S] cluster that responds to redox changes in the environment (Jakimowicz *et al.,* 2005). Such changes may alter protein conformation, and thus the activity of these proteins. It was proposed that a transient oxidative stress during growth of aerial hyphae might be sensed by WhiB, leading to changes in the expression of the WhiB-regulated genes and hence cessation of aerial hyphal growth (Chater, 2001). Unlike *whiB*, a *whiD* mutant formed normal aerial hyphae that showed, however, reduced levels of sporulation. The few spores formed were heat sensitive, lysed extensively, and were highly irregular in size and cell wall thickening. Furthermore they were usually partitioned into irregular minicompartments, devoid of chromosomal DNA (Fig 3; Fig 7; Molle *et al.,* 2000). This suggests a key role for WhiD in the activation of the later events needed for spore maturation.

Another gene playing a key role in spore biogenesis, is the *B. subtilis sigB* homologue, *sigF* (Potuckova *et al.,* 1995). A disrupted *sigF* mutant produces spores that are smaller and deformed compared to the wild type, and are characterized by thin cell walls, which render them more sensitive to detergents. Sporulation septation and spore maturation require, furthermore, transcription of the

following later genes: *ftsZ*, which specifies proper septation location through the assembling of the FtsZ rings (Flärdh *et al.*, 2000); *ssg(ABCDEFG)*, encoding a group of homologous proteins (SALPs) exclusively found in sporulating *Actinomycetes* and involved in several aspects of sporulation (spore-specific cell division, chromosome partitioning/condensation, spore wall synthesis, septum location and autolytic spore separation; Noens *et al.,* 2005); *whiE*, which includes a group of genes, encoding the PKS complex that synthesizes the grey polyketide pigment associated to the spore cell wall.

Two Component Systems (TCS) in *Streptomyces coelicolor*: The Response Regulator, RamR

Two-component signal transduction systems (TCSs), consisting of a sensor kinase (SK) and a response regulator (RR), are widely spread in *Eubacteria*, where they regulate most aspects of prokaryotic physiology. Paralogues of SK genes have also been identified in *Archaeobacteria*, fungi, protozoa and plants, suggesting that transduction pathways, similar to eubacterial TCSs, may occur in these organisms (for a review see Hutchings *et al.,* 2004). In a typical TCS, the membrane-bound sensor kinase monitors extracellular conditions and responds by changing the phosphorylation state of its cognate response regulator. Upon sensing an external signal, a SK dimer undergoes an ATP-dependent autophosphorylation of an intracellular conserved histidine residue. This phosphoryl group is then transferred to a conserved aspartate residue in the response regulator in a reaction catalyzed by its receiver domain (Fabret *et al.,* 1999). This phosphorylation changes the biochemical properties of the RR, enabling it to elicit an intracellular response. Most response regulators are DNA-binding proteins that consist of an amino-terminal regulatory receiver domain (containing the site of phosphorylation) and a carboxy-terminal DNA-binding domain that contains a helix-turn-helix motif. Other RRs, however, may exhibit an enzymatic activity triggered by phosphorylation of the N-terminal receiver domain (e.g. PleD in *Caulobacter crescentus*; Paul *et al.,* 2004) or interact with specific cellular targets, as in the case of CheY in *E. coli* that binds to components of the flagellar motor, controlling rotation of the flagellum (Welch *et al.,* 1993). Receiver domains possess a high degree of sequence and structure similarities such that residues that are important for phosphorylation and signal propagation can be easily identify (Hutchings *et al.,* 2004). These conserved residues include two adjacent aspartates near the N-terminus of the protein (D12,D13), an aspartate in the middle of the N-terminal domain (D53-54), a hydroxylated residue at position 82 (usually a serine or threonine), and a lysine residue close to position 105 (K105). The D12-D13 residues coordinate a catalytically essential Mg^{++}. D53-54 is the site of phosphorylation, and S/T82 and K105 stabilize the aspartyl-phosphate group and mediate the conformational change that results in the activation of the RR. However some RRs are atypical since they lack two or more of the conserved residues that make up the phosphorylation pocket. Others, instead, like BldM in *S. coelicolor*, are active in absence of phosphorylation, although they possess a typical phosphorylation pocket (Molle and Buttner, 2000). In some cases, the RRs and SKs of TCSs, may also undergo additional levels of

regulation through the interaction with accessory proteins. Thus, what once appeared to be relatively simple signaling pathways are likely to be more complex and to involve more than just two components. For example, in the sporulation signaling cascade of *B. subtilis* the response regulator Spo0F is subject to regulation by the RapA, B and C phosphatases, while Spo0A is regulated by the Spo0E, YnzD and YisI phosphatases (Hutchings *et al.,* 2004).

Sensor kinases and their cognate response regulators are usually encoded by genes that are cotranscribed as part of an operon. In some cases, however, SK and RR genes can be monocistronic (orphan SKs or RRs). Sequence analysis of *S. coelicolor* genome, has revealed that this soil bacterium has the potentiality to encode 84 SKs and 80 RRs (Bentley *et al.,* 2002; Hutchings *et al.,* 2004). This suggests that *S. coelicolor* might be well equipped to deal with a wide range of environmental stimuli, likely reflecting the complexity of the soil. Of these 84 SK genes, 67 are adjacent to RR genes, 17 SK genes are unpaired, and thus 13 RR genes encode orphan proteins (Hutchings *et al.,* 2004). Genetic analyses showed that TCSs and orphan RRs control several aspects of development in *S. coelicolor*: phosphate uptake (*phoPR*); cell wall damage response (*cseBC*) and vancomycin resistance (*vanRS*) (Hutchings *et al.,* 2004); antibiotic biosynthesis (*absA1-2*, *cutRS*, *redZ*; White and Bibb, 1997) and/or morphological differentiation (*osaAB*, *bldM*, *whiI*; Molle and Buttner, 2000; Ainsa *et al.,* 1999; Hutchings *et al.,* 2004).

A key response regulator that plays a central role in the activation of the morphological developmental program in *S. coelicolor* is *ramR* (see introduction chapter I; Nguyen *et al.,* 2002; Keijser *et al.,* 2002; O'Connor *et al.,* 2005). This RR is of considerable interest because it links the erection of aerial hyphae to earlier regulatory events, presumably including some mediated by *bld* gene products (Nguyen *et al.,* 2002). Indeed, overexpression of *ramR* restored AHF and SapB biosynthesis in all *bld* mutants tested, bypassing the proposed signaling cascade, as predicted by ESC experiments (see above; Fig 4; Nguyen *et al.,* 2002). Thus RamR, similarly to Spo0A in *Bacillus subtilis*, may integrate developmental, stress and/or nutritional signals to activate the growth of aerial hyphae, although the molecular mechanisms through which these signals may converge to RamR must be still defined (Nguyen *et al.,* 2002).

RamR exerts part of its effects on morphological differentiation through direct transcriptional activation of the SapB biosynthetic operon, *ramCSAB* (Fig 6; Nguyen *et al.,* 2002; O'Connor *et al.,* 2002; 2005). Because RamR is a RR, one might argue that its activity is modulated by phosphorylation

(Nguyen *et al.,* 2002). However, recent observations suggest that RamR may not have a sensor kinase. The unphosphorylated form of RamR, in fact, is a dimer that interacts tightly and cooperatively with three binding sites in the *ramC* promoter. Moreover, RamR cannot be phosphorylated with small phosphodonor molecules, suggesting that its receiver domain might lack phosphotransferase activity (O'Connor *et al.,* 2005). Nonetheless, mutations in the predicted D53 site of phosphorylation impair its function *in vivo* (Nguyen *et al.,* 2002; O'Connor *et al.,* 2005). This might be explained by the fact that the D53 residue is important for both dimer formation and DNA-binding (O'Connor *et al.,* 2005). RamR activity is in part regulated at transcriptional level. Promoter-probe analyses and S1-nuclease protection assays demonstrated that *ramR* promoter has maximal activity at 34-40 h, coincident with the onset of AHF (Nguyen *et al.,* 2002; Keijser *et al.,* 2002). In *S. griseus*, transcription of the *ramR* orthologue, *amfR*, is activated by AdpA and is therefore under control of the A-Factor signaling cascade (see above; Yamazaki *et al.,* 2003). A similar AdpA-dependent transcriptional activation of *ramR* may occur also in *S. coelicolor* as *ramR* overexpression restores AHF to the *adpA(bldH)* mutant (Nguyen *et al.,* 2003).

As RamR plays a crucial role in activating a developmental switch that leads to AHF, the main aim of my PhD work was to dissect the RamR regulon and demonstrate whether, in addition to SapB biosynthesis and transcriptional activation of the *ramCSAB* operon, RamR activated alternative and yet unidentified pathways for morphological differentiation in *S. coelicolor* (Chapter I).

In addition, as the biosurfactant activity of SapB is required to elicit AHF under certain growth and physiological conditions, another goal of my PhD work was to investigate whether the SapB-induced initiation of aerial growth was accompanied by the activation of a genetic program likely needed for differentiation of specialized spore-bearing aerial hyphae (Chapter II).

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Chapter I

The members of RamR regulon encode signaling factors that trigger morphological differentiation in the filamentous bacterium *Streptomyces coelicolor*

Salvatore San Paolo[+], Jianqiang Huang*, Stanley N. Cohen*, and Charles J. Thompson[§+]

+ Department of Molecular Microbiology, Biozentrum, Universität Basel, Basel Switzerland.

* Department of Genetics, Stanford Medical School, Stanford, CA, USA

[§] Department of Microbiology and Immunology, Life Sciences Center, University of British Columbia, Vancouver, BC, Canada

Running title: Control of *S. coelicolor* developmental genes, *rdgABKR*, by RamR

Key words: aerial hyphae, differentiation, sporulation, response regulator, histidine kinase, SapB.

Summery

The filamentous eubacterium, *Streptomyces coelicolor*, undergoes a complex growth cycle in which morphological differentiation coincides with the activation of the orphan response regulator RamR and the biosynthesis of the morphogenetic compound SapB. SapB is a lantibiotic-like molecule derived by the product of the *ramS* gene that induces aerial hyphae formation (AHF) and sporulation by breaking the aqueous tension at the surface of the vegetative mycelium. A *ramR* disrupted mutant is delayed in AHF, while constitutive overexpression of *ramR* accelerates AHF in the wild type strain and restores SapB biosynthesis and AHF to all of the *bld* mutants tested. We report the discovery of a physically distinct and distant cluster of genes, here designated as *rdg* (*ramR* dependent genes) that, like the genes of the *ram* cluster, is regulated by RamR. The genes of the *rdg* cluster include: *rdgA* (*sco4075*) and *rdgB* (*sco4074*), which encode two subunits of an ABC transporter, *rdgK* (*sco4073*), a putative histidine kinase, and *rdgR* (*sco4072*), a *ramR* paralog. DNA microarrays and quantitative real time RT-PCR analyses established the *rdg* cluster as a component of the RamR regulon. EMSA, DNaseI footprinting and S1 nuclease protection experiments confirmed that RamR exerts its effect on *rdgABKR* transcription directly, by binding to three sites in the *rdgABKR* promoter. A constructed *rdgABKR null* mutant is still able to synthesize SapB and generate aerial hyphae, but it is severely delayed and defective in sporulation. In addition, extracellular complementation and epistasis analyses in which *rdgK*, *rdgR* and the mutant D53E allele of *rdgR* were overexpressed in different developmental mutants, have revealed a role for the *rdg* operon in triggering a SapB independent sporulation pathway, downstream of RamR.

Introduction

Signal transduction pathways in prokaryotes regulate cellular functions in response to environmental and physiological cues. Two-component signal transduction systems (TCS), based on phosphorelay from a signal recognition sensor kinase (SK) to a response regulator (RR), often integrate cellular responses to such internal and external cues (Bibikov *et al.,* 1997; Jung *et al.,* 2003). Differentiating unicellular bacteria, such as *Bacillus subtilis* or *Caulobacter crescentus* use TCSs to regulate physiological and morphological switches during development (Lee Kroos and Janine R. Meddock, 2003; Stragier and Losick, 1996). Similar mechanisms regulate the developmental programs of multicellular bacteria, such as *Myxococcus xanthus* (Ellehauge *et al.,* 1998; Julien *et al.,* 2000) and *Streptomyces* (Molle *et al.,* 2000; Nguyen *et al* 2002; Hutchings *et al*., 2004).

Streptomyces coelicolor has been used as a model organism for studies of secondary metabolism and morphological differentiation. It is a soil dwelling filamentous bacterium whose genome encodes about 84 SKs and 80 RRs that it presumably uses to respond to a wide variety of environmental signals (such as nutrients and oxygen availability, cell-cell signals) that regulate developmental transitions (Hutchings *et al.,* 2004). Its developmental program begins with the germination of an exospore, which elongates and branches to form a network of multinucleoid vegetative hyphae that penetrates the solid substrate and form a tissue-like structure called vegetative or substrate mycelium. After about 40 hours of growth, the substrate mycelium generates differentiated aerial hyphae that give a characteristic white, fuzzy appearance to the colony surface. Erection of the aerial hyphae (AHF) often coincides with a transition in primary metabolism (Viollier *et al.,* 2001) and the production of antibiotics (Thompson *et al.,* 2002). The aerial hyphae eventually develop into chains of uninucleoid grey spores that provide for the dispersion and survival of the microorganism in the environment (Chater, 1993, Hopwood 1999).

Recent studies of developmental genes (Chater, 2001) have shown that they represent an interactive network. *bld* mutants are blocked in the initiation of AHF, resulting in a smooth, hairless or bald (*bld*) phenotype (Kelemen and Buttner, 1998; Chater and Horinouchi, 2003). The *whi* mutants produce aerial hyphae that are not able to complete sporulation (Chater, 1972). The block in the developmental program exhibited by the *bld* mutants is in part due to an aberrant interplay between physiological and stress response systems (Kelemen *et al.,* 2001; Viollier *et al*, 2003) often linked to impaired catabolite repression and in primary metabolism (Pope *et al.,* 1996; Süsstrunk *et al.,* 1998; Viollier *et al.,* 2001). As a result, many *bld* mutants are unable to make hydrophobic extracellular

proteins involved in morphogenesis, including chaplins (Claessen *et al.,* 2003; Claessen *et al.,* 2004; Elliot *et al.,* 2003) and SapB.

SapB is a small secreted morphogenetic peptide that can serve as a biological surfactant to release surface tension at the air-water interface, thus allowing substrate hyphae to escape the aqueous environment of the colony surface and grow upright (Tillotson *et al.,* 1998; Wosten and Willey, 2000). All *bld* mutants (except *bldF*) regain the ability to form aerial hyphae in response to externally applied SapB (Willey et al., 1991). When pairs of *bld* strains are grown close to each other on complex solid medium, one acts as the donor of a diffusible activity that induces SapB production and thereby morphological differentiation in the responsive strain (Nodwell and Losick, 1998; Nodwell *et al.,* 1999; Viollier *et al.,* 2001; Willey *et al.,* 1991; Willey *et al.,* 1993). Such extracellular complementation data have suggested a model in which each *bld* gene is directly or indirectly involved in the synthesis and response to a specific extracellular signaling molecule, defining a signaling cascade where SapB is the final product.

Kodani *et al.* (Kodani *et al.,* 2004) have reported that SapB is a lantibiotic-like peptide encoded by *ramS*, an open reading frame within the *S. coelicolor ramCSABR* gene cluster. The *ram* locus was discovered as a DNA fragment able to induce *r*apid *a*erial *m*ycelium formation when introduced on a low-copy plasmid into the closely related species *Streptomyces lividans* (Ma and Kendall, 1994). The four *ram* genes (*CSAB)* form an operon (Keijser *et al.,* 2002). SapB is derived from the prepeptide RamS through proteolytic cleavage and the introduction of four dehydroalanine residues and two lanthionine bridges. *ramC* encodes a membrane bound protein with a carboxy terminal having similarities to enzymes involved in the biosynthesis of lantibiotics, strongly suggesting its possible role in processing RamS to generate SapB (Hudson *et al.,* 2002; Kodani *et al.,* 2004). *ramA* and *ramB* encode subunits of an ABC transporter, which may be the SapB exporter (Ueda *et al.,* 2002). The fifth gene of the *ram* gene cluster, convergently transcribed to the *ramCSAB* operon, is *ramR*. RamR, a member of the NarL/FixJ family of response regulators, specifically binds to the *ramC* promoter and activates the transcription of the *ramCSAB* operon (Nguyen *et al.,* 2002; Keijser *et al.,* 2002; O' Connor *et al.,* 2002; O' Connor *et al.,* 2005).

Genetic studies have shown that RamR is a key regulatory protein for the activation of the morphological differentiation program of *S.coelicolor*. A *ramR* mutant is blocked in SapB production and delayed in AHF, while its overexpression restored SapB biosynthesis and AHF in all *bld* mutants tested (Nguyen *et al.,* 2002). RamR overexpression in the wild type strain accelerated development on solid

medium, triggered SapB biosynthesis on media where it is not normally made, and determined colony shape on semisolid media. RamR overexpression was also able to suppress the developmental arrest of a citrate synthase mutant (*citA*) (Viollier *et al.,* 2001) that was unable to erect aerial hyphae due to the accumulation of organic acids. It restored AHF without suppressing metabolic defects, thus demonstrating that its activity can uncouple morphological differentiation from metabolism (Nguyen *et al*., 2002).

In the present study we have characterized the RamR regulon using microarray and quantitative real time RT-PCR analyses, demonstrating that RamR plays a key regulatory role in development by directly activating transcription of a group of four previously uncharacterized *R*amR *d*ependent *g*enes (*rdgABKR*). These form an operon reminiscent of the *ram* locus, encoding two subunits of an ABC transporter, a histidine kinase and a response regulator with 39% identity with RamR. While a *rdgABKR null* mutant strain was able to produce SapB and generate aerial hyphae, it was severely delayed and affected in sporulation. Furthermore, extracellular complementation and epistasis analyses in which *rdgK*, *rdgR* and the D53E mutant allele of *rdgR* were overexpressed in *bld* and *ram* mutants have revealed a role for this operon in triggering the sporulation program of *S. coelicolor* by activating a SapB independent pathway of aerial hyphae formation downstream of RamR.

Results

Genome wide analyses of expression profiles in the *ramR* mutant and *ramR* overexpressing strains compared to the congenic wild type parental strain J1501

DNA microarrays and quantitative real time RT-PCR analyses (qRT-PCR) were used to compare J1501 to congenic derivatives in which *ramR* was disrupted (BZ5; Nguyen *et al.,* 2002) or overexpressed (J1501/pKN22 or RamR^{++}; Nguyen *et al.,* 2002). Synchronously pregerminated spores from the three strains were inoculated on cellophane discs placed on the surface of R2YE agar medium. While the three strains had indistinguishable growth rates, the kinetics of their developmental programs were quite different (Fig. 1A, B). The RamR^{++} formed aerial hyphae at 24 h, followed by J1501 at 36 h; sparse aerial hyphae appeared on the *ramR* mutant only at 60 h. In J1501 or RamR^{++}, the onset of aerial hyphae was coincident with SapB biosynthesis (Fig. 1B).

Since RamR drives transcription of the *ramCSAB* operon, we used these *ram* genes to validate our experimental approach. Microarray and qRT-PCR kinetic analyses confirmed that all of the *ram* genes were upregulated in RamR^{++} and downregulated in the *ramR* mutant throughout development (Fig. 2, and data not shown). *ramS* was the only small gene of the genome exhibiting a transcription profile corresponding to the kinetics of SapB biosynthesis, providing additional genetic evidence that SapB is encoded by *ramS* (Kodani *et al.,* 2004). In particular, qRT-PCR analyses of J1501 showed that *ramR* transcription was maximal at 34 h; *ramS* transcription was maximal at 40 h, coincident with SapB biosynthesis and AHF (compare Fig. 3A, with Fig. 1B). In RamR^{++}, both *ramR* and *ramS* transcription were maximally upregulated (relative to J1501) at 24 h, coincident with SapB biosynthesis and aerial growth (compare Fig. 3B and Fig. 2 with Fig. 1B).

***sco4072-4075 (rdgABKR)* is a RamR-dependent operon.**

Microarray analyses revealed another group of genes, *sco4072*, *sco4073*, *sco4074* and *sco4075*, which were upregulated in RamR^{++} throughout development. The expression profile patterns of these genes coclustered with the *ram* genes (Fig. 2). These observations suggested that they could be direct or indirect targets of RamR. Unlike the *ramCSAB* genes, however, these microarray analyses did not reveal decreased transcription of the *sco4072-4075* gene cluster in the *ramR* mutant strain relative to J1501. A more sensitive and accurate method of quantification based on qRT-PCR demonstrated that

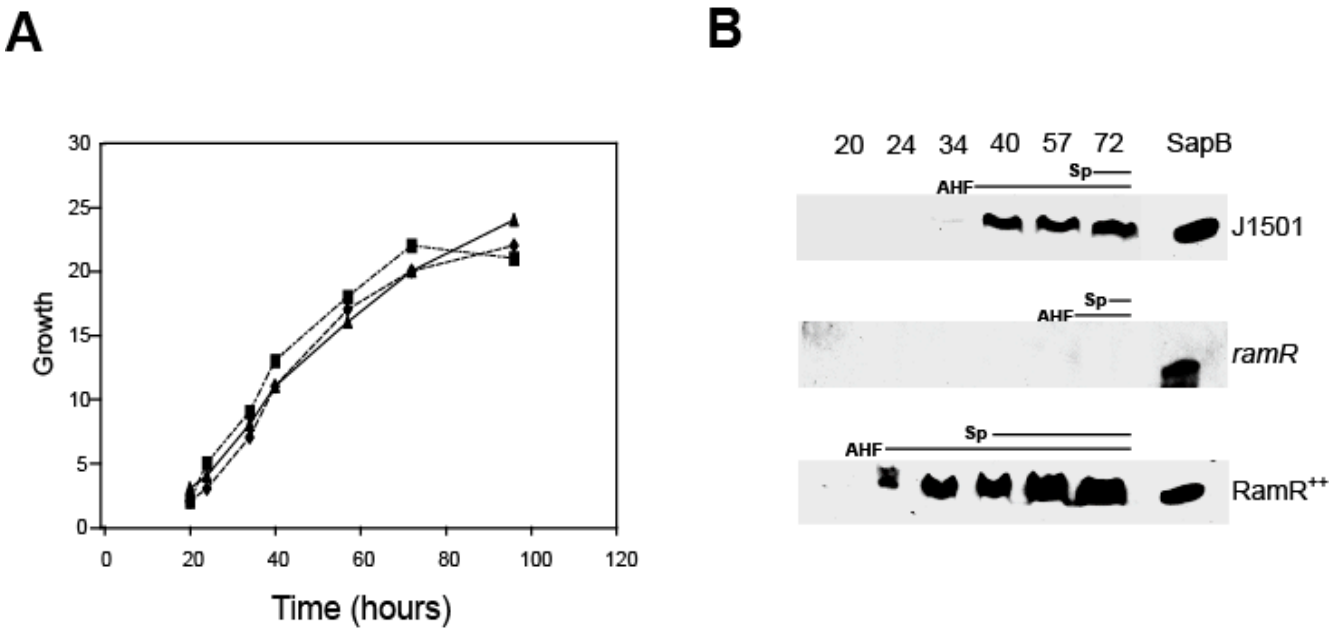

Fig. 1. Growth and differentiation of *Streptomyces coelicolor* J1501, *ramR* mutant and RamR^{++}.
(A) Pregerminated spores from J1501 (▲), *ramR* mutant (◆), and RamR^{++} (■) were inoculated on cellophane discs on R2YE medium and incubated at 30°C. Growth was calculated as mg of dry mycelium. All three strains displayed the same growth rate during development.
(B) Mycelium- and spore associated proteins were extracted from R2YE solid cultures, grown as described in A. AHF and Sp indicate phases of aerial hyphae formation and sporulation. SapB was not detected on Western blots of the *ramR* mutant even though it eventually formed aerial hyphae and sporulated at around 60 h. Both J1501 and RamR^{++} formed aerial hyphae coincident with SapB biosynthesis.

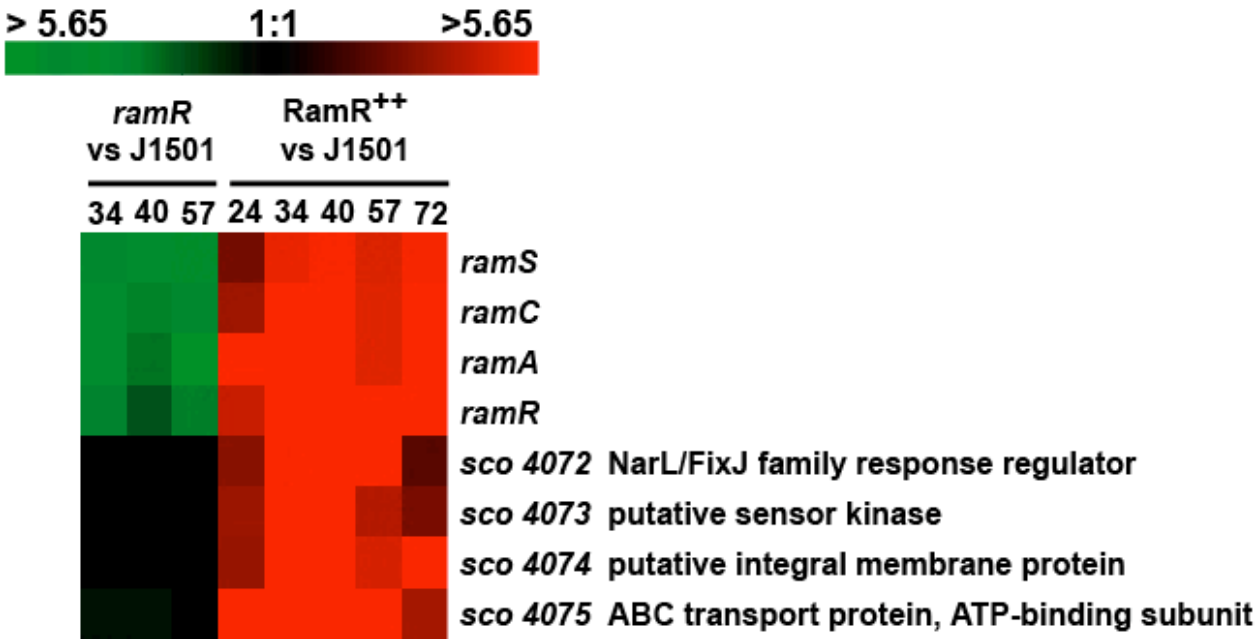

Fig. 2. DNA chip analyses identify *ramR* dependent genes. Profiles indicate the ratios of gene expression in the *ramR* mutant and RamR^{++} strains relative to J1501. RNA was extracted at the indicated time points from the solid R2YE cultures described in Fig. 1A. For microarray experiments, Cy5-dCTP (red)-labeled cDNA samples from either *ramR* mutant or RamR^{++} were hybridized with the same time point Cy3-dCTP (green)-labelled cDNA samples of J1501. Genes were clustered hierarchically according to similarity in their expression profiles (Huang *et al.,* 2001); rows correspond to individual genes and columns correspond to successive time points. The change in transcript abundance for each gene is displayed on a color scale, in which color saturation represents the magnitude of the difference of RNA abundance. The brighter red shades represent higher transcript abundance and brighter green shades represent lower transcript abundance relative to J1501. Black indicates an equal amount of RNA abundance in the two strains, and grey represents absence of data. Ratios of genes with multiple spots on the array were averaged.

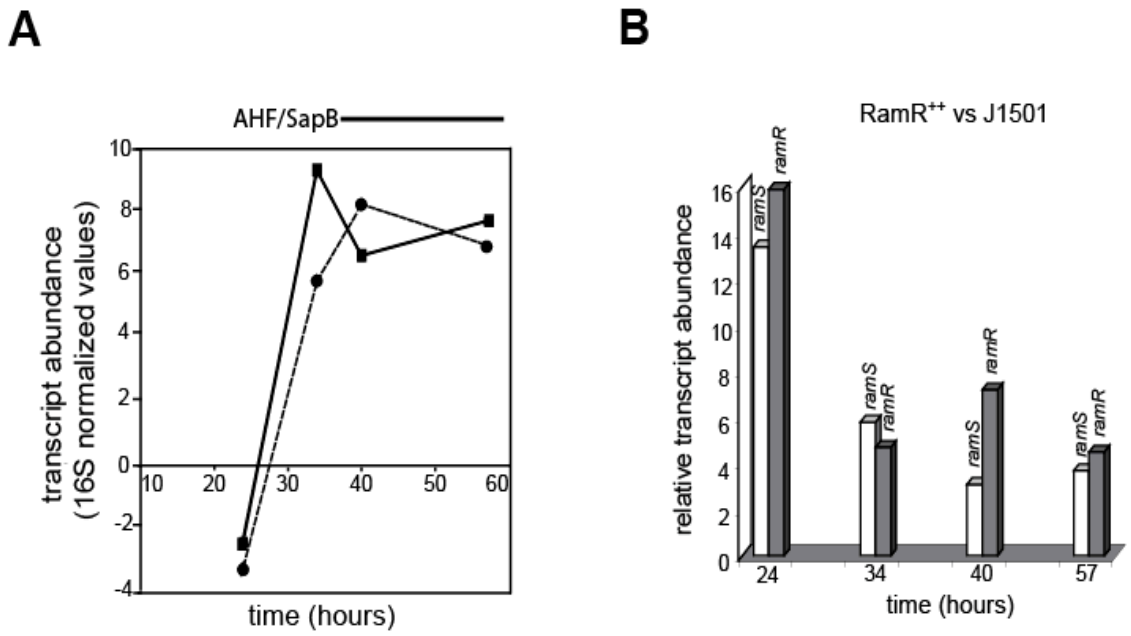

Fig. 3. Developmental regulation of *ramR* and *ramS* in *S. coelicolor* J1501.
(A) Kinetics of transcript abundance of *ramR* (■) and *ramS* (●) in the wild type strain of *S.coelicolor,* J1501. RNA samples were isolated at the indicated time points from the same solid R2YE cultures described in Fig. 1A and analysed by qRT-PCR. The amount of each cDNA tested was internally normalized to the 16S rDNA and transcript abundance for each gene was displayed as $\log_2$ of normalized values. A maximal level of the *ramR* transcript was registered at 34 h of development, followed by *ramS* transcript that was maximal at 40 h of growth, coincident with SapB production and AHF (compare with 1B).
(B) Expression profiles of *ramR* and *ramS* in RamR^{++} relative to the congenic parent strain, J1501. RNA samples were isolated from the solid R2YE cultures described in A and analysed by qRT-PCR. The levels of gene expression in each sample were calculated as $\log_2$ of the ratios (RamR^{++} relative to J1501) after normalization to the 16S rDNA. Both *ramR* and *ramS* are constitutively upregulated in RamR^{++} over time with an expression profile coinciding with SapB biosynthesis (compare with Fig 1B and 2).

transcription of these genes was dependent on RamR activity (Fig. 4A) and was developmentally regulated (Fig. 4B).In the wild type strain, J1501 (Fig. 4B), *sco4072, sco4074* and *sco4075* transcripts exhibited a peak in transcript abundance at 34 h, coincident with the expression of *ramR* and the onset of AHF. Furthermore all four *sco4072-4075* genes were constitutively upregulated in RamR^{++} and downregulated in the *ramR* mutant (Fig. 4A and data not shown). Similar results were observed for *ramR* (Fig. 4A) and *ramCSAB* genes (data not shown), serving as internal controls.

To extend the correlation of RamR activity with transcription of *sco4072-4075* , we performed additional qRT-PCR analyses using RNA extracted from liquid (J medium) cultures of J1501, RamR^{++} and *ramR* mutant extracted at 24 h and 30 h of development at 30°C, during the mid log phase. Once again, the level of *sco4072-4075* transcription correlated with *ramR* transcription (RamR^{++} vs J1501, *ramR* mutant vs J1501 and RamR^{++} vs *ramR* mutant; Fig. 5).

These results showed that *sco4072-4075* were *R*amR *d*ependent *g*enes (*rdg*). This cluster includes: *rdgA* (*sco4075*) and *rdgB* (*sco4074*), which encode two putative subunits of an ABC transporter, *rdgK* (*sco4073*), encoding a putative histidine kinase and *rdgR* (*sco4072*) that encodes a response regulator of the NarL/FixJ family with 39% identity with RamR (Fig. 6A). These *rdg orfs* are in the same orientation, with a partial overlap between the stop codon of *rdgA* and the start codon of the downstream gene *rdgB* (Fig. 6A). To test whether the four *rdgABKR* genes formed an operon, RT-PCR experiments were done with specific pairs of primers to amplify overlapping products spanning from the last 100-150 bp of the 3' end of one gene to the first 100-150 bp of the 5'-end of the downstream gene (Fig. 6A). When RNA extracted from solid cultures of J1501 at 40 h and 60 h of development was used to generate cDNA templates, specific amplification products corresponding to cotranscription of either *rdgA-rdgB* or *rdgB-rdgK* or *rdgK-rdgR* were detected at both time points tested, demonstrating that the four *rdgABKR* genes were cotranscribed in a polycistronic mRNA (Fig. 6B; data not shown). Gene expression studies suggested that RamR may activate transcription of the *rdgABKR* operon by binding directly to the region upstream of *rdgA.*

Recombinant RamR binds specifically to three sites in the intergenic region upstream of the *rdgABKR* operon.

Recombinant unphosphorylated RamR, able to bind to three sites on the *ramC* promoter (Nguyen *et al.,* 2002; O'Connor *et al.,* 2005) was tested for its ability to bind to specific sites on the intergenic region

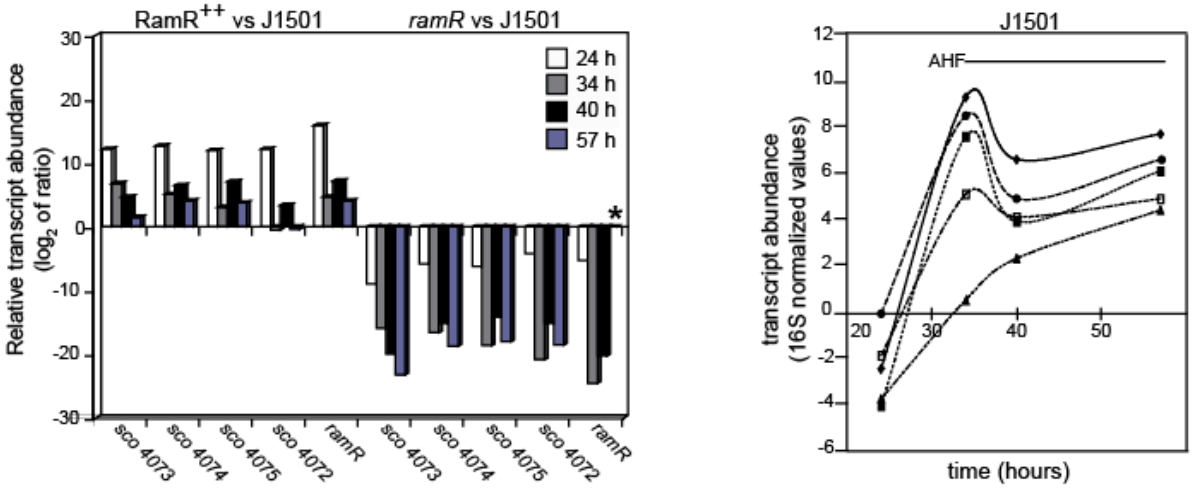

Fig. 4. RamR dependent transcription of the *sco4072-4073* gene cluster.
(A) Quantitative real time RT-PCR analyses examining transcriptional profiles of selected genes from Fig. 2, confirmed their dependence on RamR activity. The levels of gene expression in each sample were calculated as $\log_2$ of the ratios (RamR^{++} or *ramR* mutant relative to J1501) after normalization to the 16S rDNA. The asterisk indicates absence of signal in the *ramR* mutant even after 40 cycles of amplification. The RNA used for these experiments was the same as was used for the microarray analysis shown in Fig. 2.
(B) Kinetics of transcript abundance of *ramR* (◆), *sco4072* (●), *sco4073* (▲), *sco4074* (■) and *sco4075* (□) in the wild type strain of *S. coelicolor,* J1501. RNA samples used for this analysis were the same as those used for the experiments described in Fig. 1 and Fig 2. The amount of each cDNA tested was internally normalized to the 16S rDNA and transcript abundance for each gene was reported as $\log_2$ of normalized values. All of the genes tested, with the exception of *sco4073*, displayed transcriptional kinetics very similar to *ramR* with maximal transcript abundance at 34 h, coincident with SapB biosynthesis and AHF (compare with Fig. 1B).

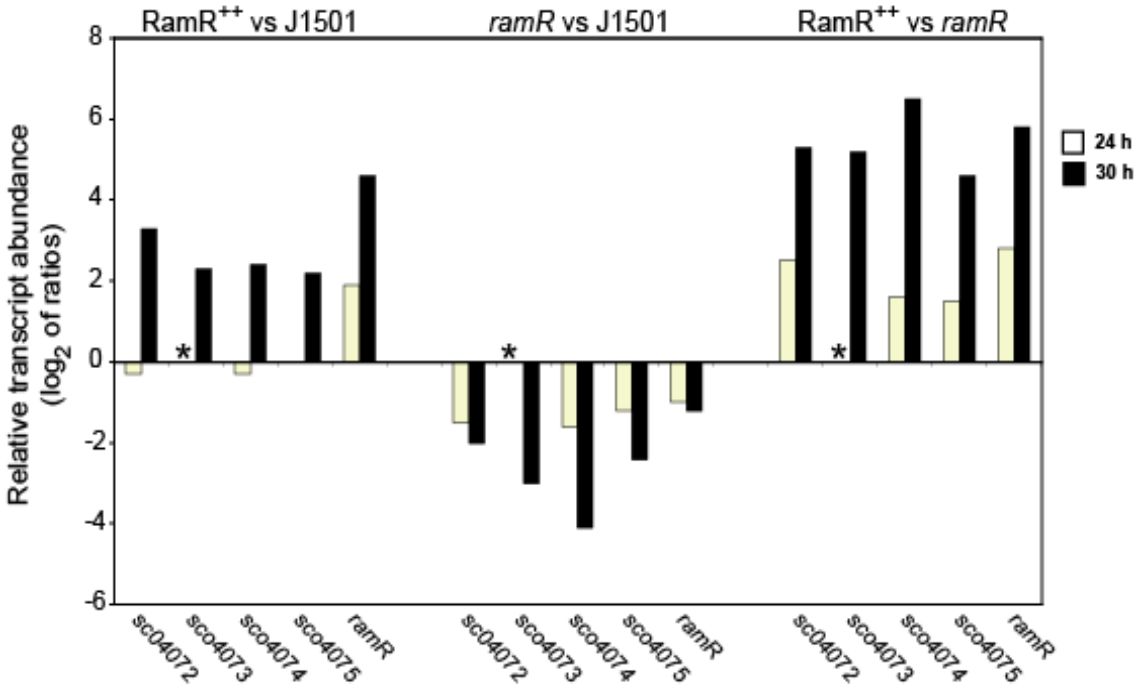

Fig. 5. Quantitative real time RT-PCR analyses examining the expression profiles of selected genes from Fig. 2 in liquid J medium cultures. RNA was extracted from J1501, *ramR* mutant and RamR^{++} at 24h and 30 h of development in liquid J medium. The extent of gene expression in each sample was calculated as $\log_2$ of the ratios (RamR^{++} or *ramR* mutant relative to J1501) after normalization to the 16S rDNA. Also in these conditions where the three strains of *S. coelicolor* do not differentiate into spores, the expression of all four genes of the *sco4072-4075* locus was dependent on *ramR* expression. The asterisk indicates absence of signal in the *ramR* mutant and J1501 even after 40 cycles of amplification.

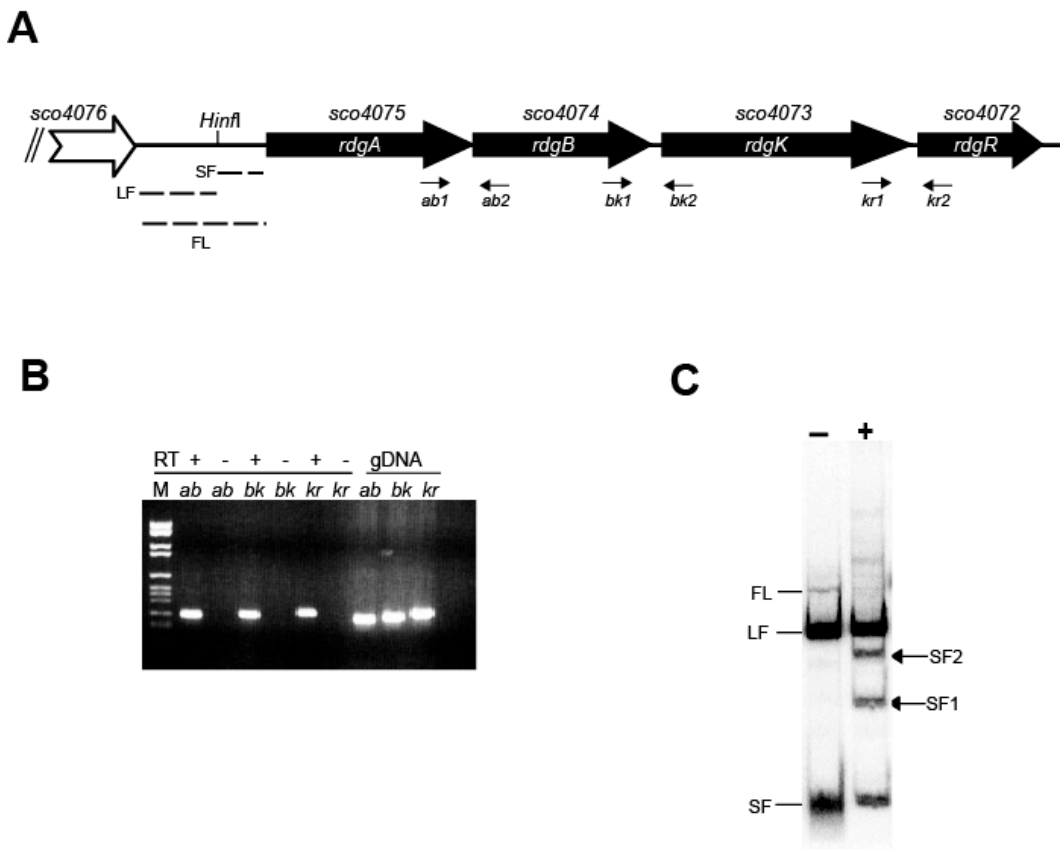

Fig. 6. Genetic organization and RamR dependent transcription of the *Streptomyces coelicolor rdgABKR* operon.

(A) Genetic organization of the *S. coelicolor sco4072-4075* operon. The orientation of the four *sco4072-4075* genes is indicated by black arrows. The white arrow corresponds to the upstream open reading frame. Arrows (→) show the orientation and location of the primer pairs used for the cotranscription analyses of the *rdgABKR* genes. Dashed lines correspond to either the full length (FL), the 180 bp *Hinf*I fragment (small, SF), or the 380 bp *Hinf*I fragment (long, LF). These probes were made by PCR-radiolabeling the 560 bp intergenic region upstream of *rdgA* (full length, FL) followed by digestion with the restriction enzyme *Hinf*I (see Experimental Procedures).

(B) Cotranscription analysis of the four *rdgABKR* genes in the *S. coelicolor* strain, J1501. RNA was extracted from mycelium harvested after 60 h (see Experimental Procedures). RT-PCR reactions containing primer pairs *ab1* + *ab2*, *bk1* + *bk2* and *kr1* + *kr2* (as shown in panel C) were used to assess cotranscription of *rdgA*-*rdgB* (*ab*), *rdgB*-*rdgK* (*bk*) and *rdgK*-*rdgR* (*kr*) respectively in presence (+) or absence (-) of reverse transcriptase (RT). Genomic DNA (gDNA) was used as positive control for the PCR conditions. PCR products were separated on 2% agarose gel in TBE 1X buffer. The integrity of the cDNA samples was confirmed with primer pair specific for the *hrdB* gene of *S.coelicolor* (data not shown).

(C) Gel mobility shift assay showing RamR binding to an operator region upstream of *rdgA*. The full length intergenic region upstream of *rdgA* (FL) was radiolabelled and digested with *Hinf*I to generate a long fragment (LF) and a small fragment (SF) probe, as shown in panel C. Recombinant RamR bound specifically to the SF probe forming two stable complexes, SF/C1 and SF/C2. Reactions contained the radiolabelled probe mix with (+) or without (-) purified recombinant RamR (100 nM).

upstream of the *rdgABKR* operon. To determine the minimal fragment that could be bound by RamR, the 560 bp intergenic region (FL) upstream of the putative translational start site of *rdgA* was digested with *Hinf*I to generate two fragments (see Experimental Procedures): the 180 bp SF-probe, immediately upstream of *rdgA*, and the 380 bp LF-probe (Fig. 6A). Electrophoretic mobility shift assay (EMSA) showed that recombinant RamR bound specifically to the SF-probe where it formed two stable complexes (SF/C1 and SF/C2 in Fig. 6C). This suggested that binding sites were located within a 180 bp sequence upstream of the annotated start codon of *rdgA*.

To confirm the specificity of RamR DNA binding, the 180 bp (S) *Hinf*I restriction fragment was end radiolabeled with P^{32} on its sense strand (SF*-probe in Fig. 7A) and used both in EMSA and in DNaseI footprinting experiments (Fig. 7B, C). EMSA of the titrated protein confirmed its ability to bind, in a concentration dependent manner, to at least two operator sites within the SF*-probe with an average binding coefficient (Kd) of about 8.4 x 10^{-8} M (Fig. 7B). In order to determine the precise location of the binding sites of RamR on the SF*-probe, DNaseI protection assays were done using the same reaction conditions as for EMSA. Three distinct regions (A1, A2 and A3), each one spanning about 12 bp, were protected from DNaseI cleavage (Fig 7A, C and 9A). Site A1 and A2 were strongly protected at 60 nM of RamR, whilst A3 was similarly protected at about double that concentration (130 nM), thus implying that RamR binds first to A1 and A2 (corresponding to the SF*/C1 complex in fig. 7B) and then to A3 (the SF*/C2 complex in Fig. 7B) which has a weaker binding affinity.

RamR has similar binding topologies on both the *rdgABKR* and *ramCSAB* operons.

In order to better understand transcription regulation of the *rdgABKR* operon, we determined the transcription initiation site(s) for *rdgA*, the first gene of the operon. High-resolution S1 nuclease mapping experiments were carried out with RNA extracted from J1501, $RamR^{++}$ and *ramR* mutant after 24 h and 60 h of development on R2YE medium. A single transcription initiation site was found in both J1501 at 60 h and $RamR^{++}$ at 24 h and 60 h of development, whereas no transcriptional initiation could be detected in the *ramR* mutant strain at either these two time points (Fig 8).

The RamR protected regions A1 and A2 are localized at -75 to -61 and at -58 to -49 nt with respect to the transcriptional initiation site and the weaker protected A3 site at -37 to -28 nt. Thus, the A1-A2 operator region is upstream of the predicted promoter recognition hexamer centered at -35, while A3 partially overlaps with it (Fig 9A).

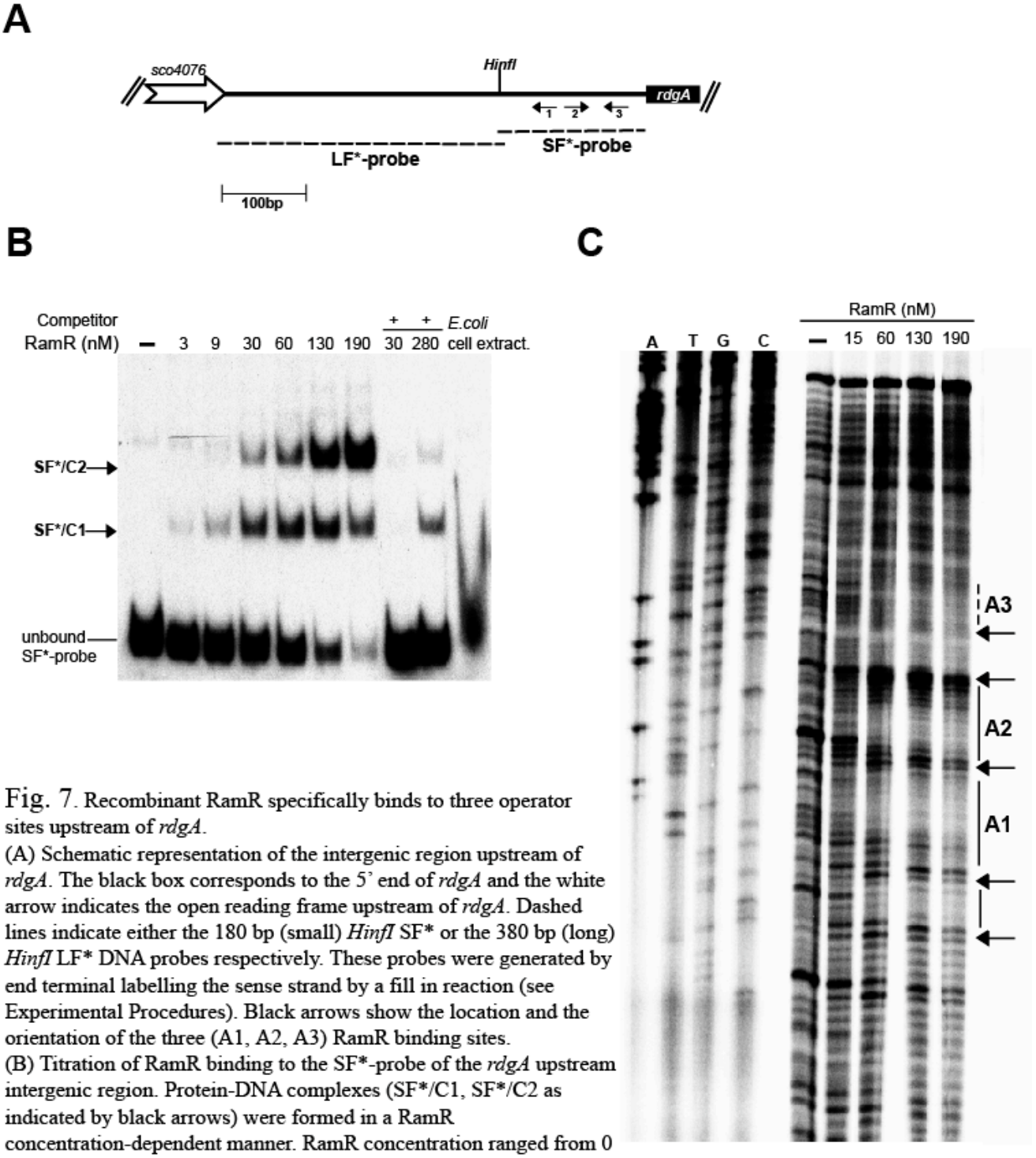

Fig. 7. Recombinant RamR specifically binds to three operator sites upstream of *rdgA*.
(A) Schematic representation of the intergenic region upstream of *rdgA*. The black box corresponds to the 5' end of *rdgA* and the white arrow indicates the open reading frame upstream of *rdgA*. Dashed lines indicate either the 180 bp (small) *HinfI* SF* or the 380 bp (long) *HinfI* LF* DNA probes respectively. These probes were generated by end terminal labelling the sense strand by a fill in reaction (see Experimental Procedures). Black arrows show the location and the orientation of the three (A1, A2, A3) RamR binding sites.
(B) Titration of RamR binding to the SF*-probe of the *rdgA* upstream intergenic region. Protein-DNA complexes (SF*/C1, SF*/C2 as indicated by black arrows) were formed in a RamR concentration-dependent manner. RamR concentration ranged from 0 nM (—) to 190 nM in the presence of 0.06 pmol of SF*-probe.
The addition of 500 ng of unlabeled specific competitor DNA to a reaction mix containing 30 nM of protein completely abolished the formation of the protein- labeled DNA complexes; it strongly reduced the protein-DNA complex formation when a higher concentration of RamR (280 nM) was used. No protein-DNA complexes could be visualized when 14 ng total cell extract from the plasmid free *E. coli* host was added to the reaction mix.
(C) DNaseI footprinting of RamR binding to (SF*-probe) the *rdgA* upstream intergenic region. DnaseI footprints were performed with increasing concentration of RamR protein, ranging from 0 nm (—) to 190 nM. Areas of strong protection are indicated by solid lines (A1, A2) while the third weakly protected area (A3) is indicated by a dashed line. Black arrows indicate the location of the DNaseI hypersensitive sites. A, T, G, C correspond to the sequence ladder.

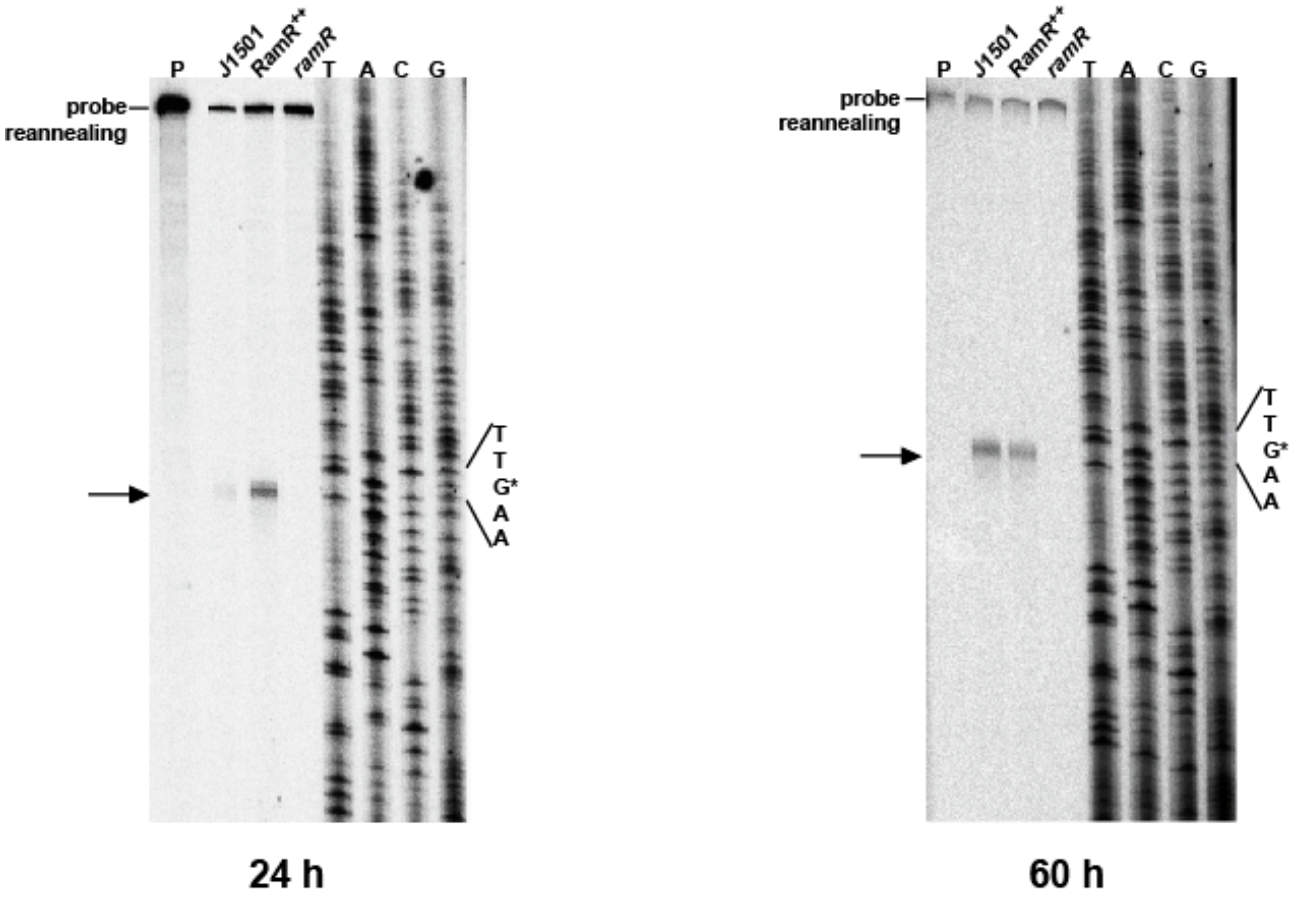

Fig. 8. High resolution S1 nuclease mapping of the 5' end of an *rdgA* transcript in J1501, RamR^{++} and the *ramR* mutant strains of *S. coelicolor*. RNA was isolated from the three strains after 24 h or 60 h of growth on cellophane discs on solid R2YE medium. Black arrows indicate the position of the S1 protected fragment and the asterisk indicates the +1 nucleotide corresponding to the predicted transcriptional initiation site. Lane P corresponds to the probe alone hybridized in presence of 45 μg of tRNA which was used as negative control. Lanes T, A, C, G represent a dideoxy sequence ladder of the same region generated with primer prdg-S1. A 16S rRNA specific probe and *rrnA* specific primers were used in either high-resolution S1 nuclease mapping or RT-PCR experiments to confirm the integrity of the RNA samples and as loading control (not shown). The same results were obtained when another internal standard, *hrdB*, was used for normalization (data not shown).

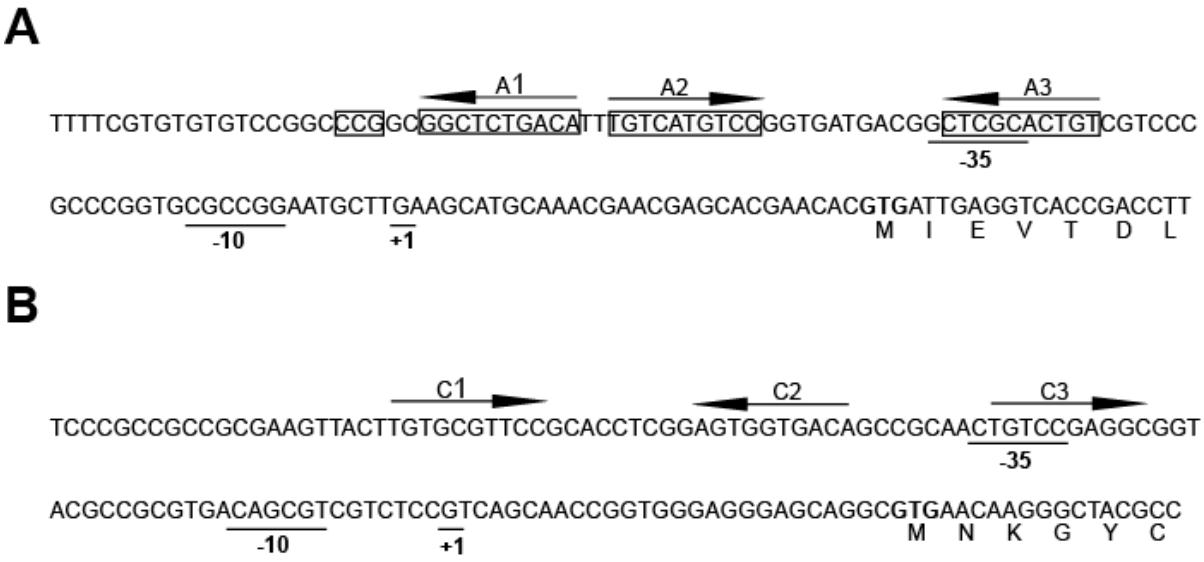

Fig. 9. Nucleotide sequence of the sense strand of the *rdgABKR* and *ramCSAB* promoter regions. (A) The transcriptional initiation site is underlined and indicated as +1. The -10 and -35 sequences are also underlined while the RamR DnaseI protected regions, A1, A2 and A3, (see also Fig. 7C) are in boxes. Black arrows, above the nucleotide sequence, indicate the orientation of the RamR binding sites as determined by the RamR consensus sequence. The GTG start codon of *rdgA* is indicated in bold.
(B) Nucleotide sequence of the *ramCSAB* promoter region. The transcriptional initiation site is underlined and indicated as +1. The predicted -10 and -35 RNAP binding hexamers are also underlined. Black arrows, above the nucleotide sequence, indicate the orientation and location of the consensual RamR binding sites (C1, C2 and C3). In bold is the GTG start codon of *ramC*.

Sequences of the small *HinfI* fragment of the *rdgABKR* promoter and of the *ramC* promoter were analyzed together or independently by *in silico* analyses (http://bioweb.pasteur.fr/seqanal/motif/meme/meme.html) to identify common motifs on the two promoter regions. These were compared with the results of the High-resolution S1 mapping, gel mobility shift assays and DNaseI footprinting experiments to define a consensus sequence for RamR binding: TGTC(A/C)(G/C)RNYC. In agreement with what was reported by O'Connor *et al.,* 2005, three sites (C1, C2, C3 in Fig. 9B) matched the RamR binding consensus sequence in the *ramC* promoter region.

The *ΔrdgABKR null* mutant is delayed in sporulation.

Previous studies have shown that *ramR* and the genes of the *ramCSAB* operon play an important role for the activation of the aerial hyphae formation program in *S. coelicolor* on the complex medium R2YE (Nguyen *et al.,* 2002; O'Connor *et al.,* 2002; Keijser *et al.,* 2002). To investigate whether the *rdgABKR* operon could also play a role in development, the entire operon was deleted in the wild type strain, J1501, using a suicide vector pSKΔ, (derived from pSET151-see Experimental Procedure). Unlike the *ramC*, the *ramS* and the *ramR* mutants, the *ΔrdgABKR null* mutant did not have a *bld* phenotype, nor was it delayed in aerial hyphae formation on R2YE. Furthermore, no obvious morphological phenotype were observed on MM medium; pigmented antibiotic biosynthesis was unaffected.

However when pregerminated spores from the *ΔrdgABKR null* mutant strain were inoculated on R2YE plates, a significant delay and reduction in sporulation was observed visually or by scanning electron microscopy (Fig. 10A). Morphological differentiation of the mutant was fully restored by introducing the *rdgABKR* operon using the integrative vector pHM11a (*ΔrdgABKR*/pSNP6; Fig. 10B). After three days of development, both the wild type (data not shown) and the *ΔrdgABKR*/pSNP6 (Fig. 10B) strains were covered with abundant straight or loosely coiled spore chains with several tens of spores. In contrast, the *ΔrdgABKR null* mutant was covered with irregular undifferentiated aerial hyphae that were often branched, having few septal constrictions. Unlike the classical sporulation defect of *whi* mutants (Chater, 1972; Flärdh *et al.,* 1999), the *ΔrdgABKR* mutant produced some gray pigment and a few spore chains after a delay of two days (compared to the J1501 strain; Fig. 10).

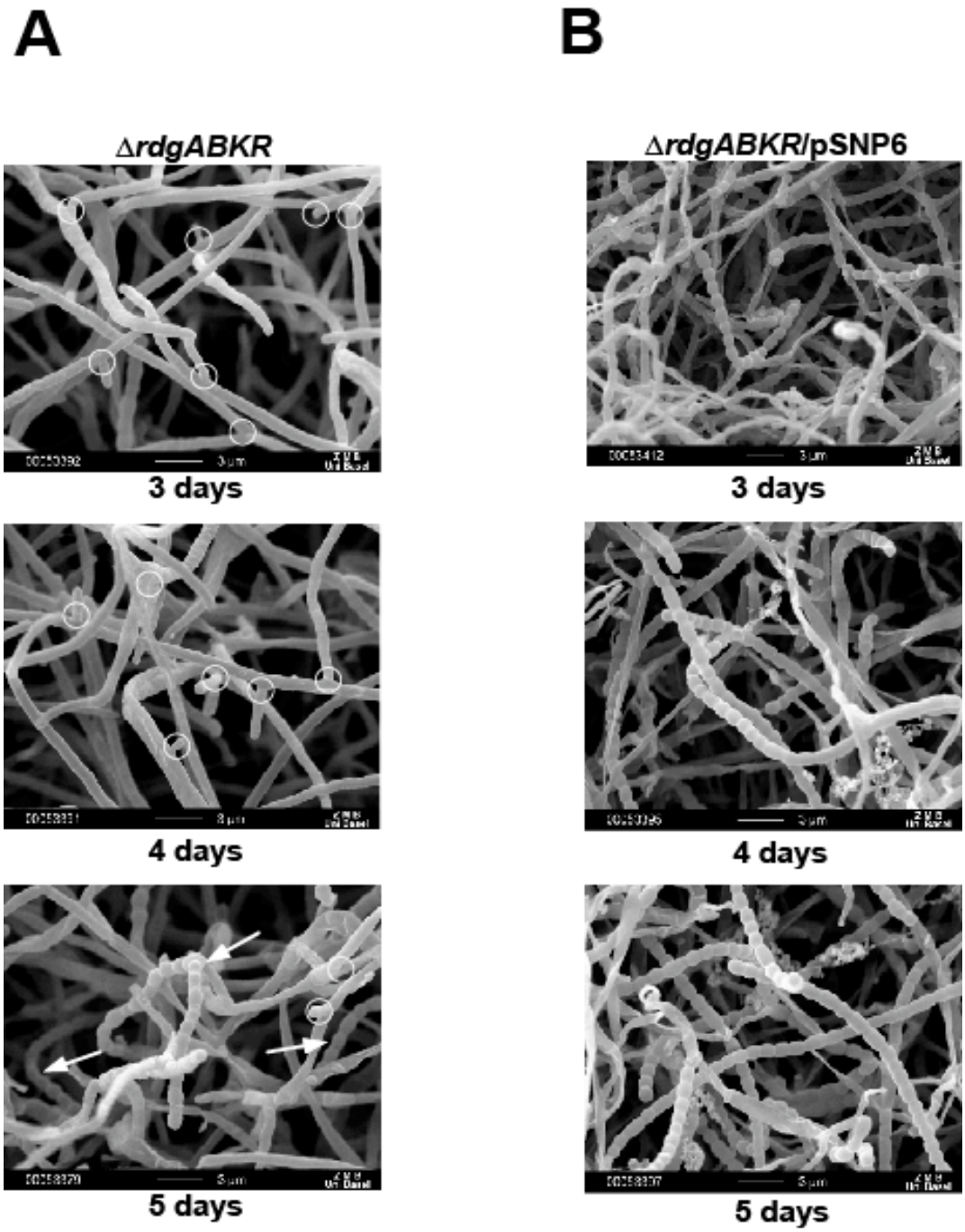

Fig. 10. Developmental defects of the *ΔrdgABKR null* mutant strain (A) compared to the complemented *ΔrdgABKR*/pSNP6 strain (B) of *S. coelicolor*. Cultures were incubated at 30°C on R2YE plates and scanning electron micrographs were taken at the indicated time points. In contrast to the complemented *ΔrdgABKR*/pSNP6 strain that was covered with abundant spore chains after 3 days of development, the *ΔrdgABKR null* mutant exhibited a severe delay in sporulation generating long, loosely coiled unseptated aerial hyphae that often branched (white circles). Few normal spore-bearing aerial hyphae were observed. Hyphae with indentations that might reflect initiation of septation were observed only after 5 days of development (white arrows).

Overexpression of the D53E mutant allele of *rdgR* restored aerial hyphae formation and sporulation in developmental mutants in the absence of SapB

rdgK and *rdgR* encode a putative histidine kinase and a response regulator. Epistasis experiments were done to learn more about the role of these regulatory genes in aerial hyphae formation and differentiation. *rdgK* and/or *rdgR* were overexpressed in the developmental mutant strains *ramR*, *ramS* and *bldK* (from the constitutive *ermE* promoter in the integrative expression vector pHM11a (Motamedi *et al.,* 1995) (pSNP1, pSNP2, pSNP3; see Experimental Procedures). Overexpression of *rdgK* or *rdgR* genes (pSNP1 or pSNP2) weakly induced AHF within four days of development on R2YE medium only in the *ramR* mutant (Fig. 11A). However, when both *rdgK* and *rdgR* where co-overexpressed (using pSNP3), AHF and sporulation could be fully restored in all of the developmental mutants tested (Fig. 11A, B; data not shown).

The observation that AHF could be efficiently restored only by co-overexpression of both *rdgK* and *rdgR*, suggested that to be fully active RdgR required phosphorylation, probably by its genetically linked histidine kinase, RdgK. The predicted RdgR protein has a typical FixJ C-terminal DNA binding domain and an N-terminal phosphorylation motif (Hutchings *et al.,* 2004) (D53). To test whether phosphorylation of the D53 residue could play an important role for the *in vivo* function of RdgR, D53N and D53E alleles of *rdgR* were constructed, cloned into the integrative vector pHM11a (pSNP4 and pSNP5), and introduced in representative developmental mutants of *S. coelicolor* (Fig. 12A, B). Studies of other response regulators predict that RdgR(D53N) would not be activated by phosphorylation, while RdgR(D53E) would be in a constitutive active conformation (Parkinson and Kofoid, 1992). Supporting those predictions, overexpression of the mutant D53E allele of *rdgR* (pSNP5) restored growth of aerial hyphae to the *ramR*, *ramS* and *bldK* mutants in three days and to the *bldJ*, *bldH* and *ramC* mutants after five days of development on R2YE medium (Fig. 12B). Sporulation, as indicated by the production of the grey pigment, was observed in all the RdgR(D53E)-rescued *ram* and *bld* mutants (except in *ramC*) after a prolonged incubation (5-10 days at 30°C; data not shown; Fig. 12B). However, unlike *ramR* overexpression (Nguyen *et al.,* 2002), *rdgR(D53E)*(pSNP5) did not accelerate development in the wild type strain J1501 (Fig. 12A). Rescue of AHF was not observed when the mutant D53N allele of *rdgR* (pSNP4) was overexpressed using the same vector in same developmental mutant backgrounds (Fig. 12A; data not shown).

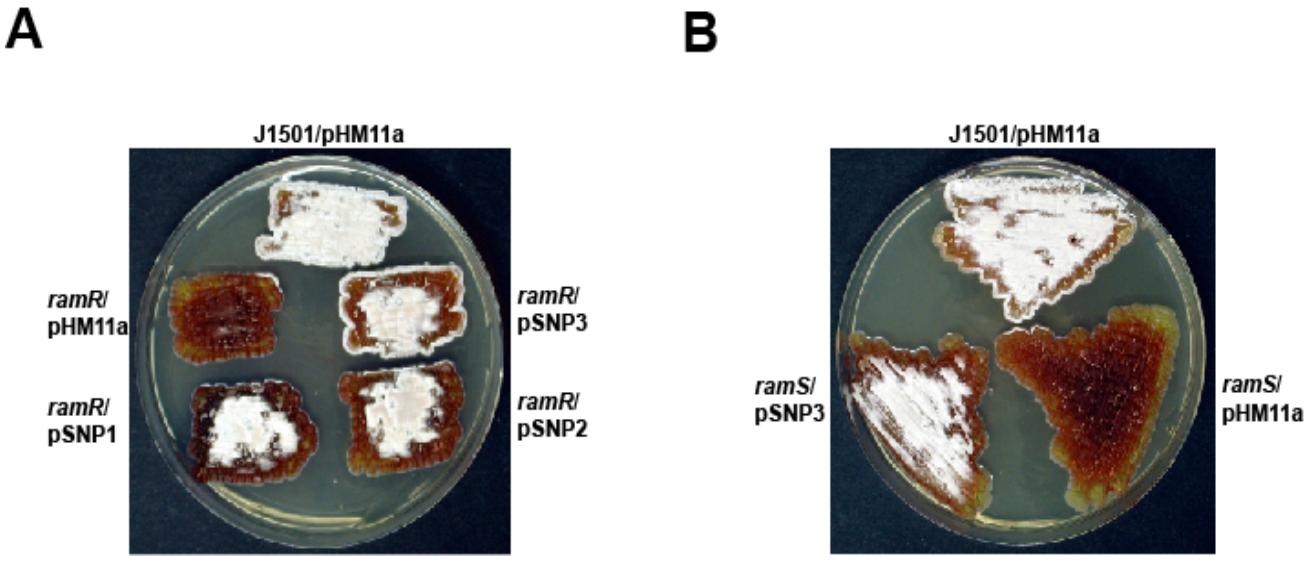

Fig. 11. Suppression of the morphological defect in the *ramR* and *ramS* mutant strains overexpressiong *rdgK*, *rdgR* and *rdgKR*.
rdgK, *rdgR*, *rdgKR* cloned into the integrative expression vector, pHM11a (pSNP1, pSNP2, pSNP3 respectively), were introduced into the *ramR* (A) or the *ramS* (B) mutants by conjugation. Suppression of the morphological defect by induction of AHF could be observed in *ramR* mutant overexpressing either *rdgR* (pSNP5) or *rdgK* (pSNP2) or *rdgKR* (pSNP3) during development on R2YE plates. AHF was restored only in the *ramS* mutant overexpressing *rdgKR* (pSNP3).

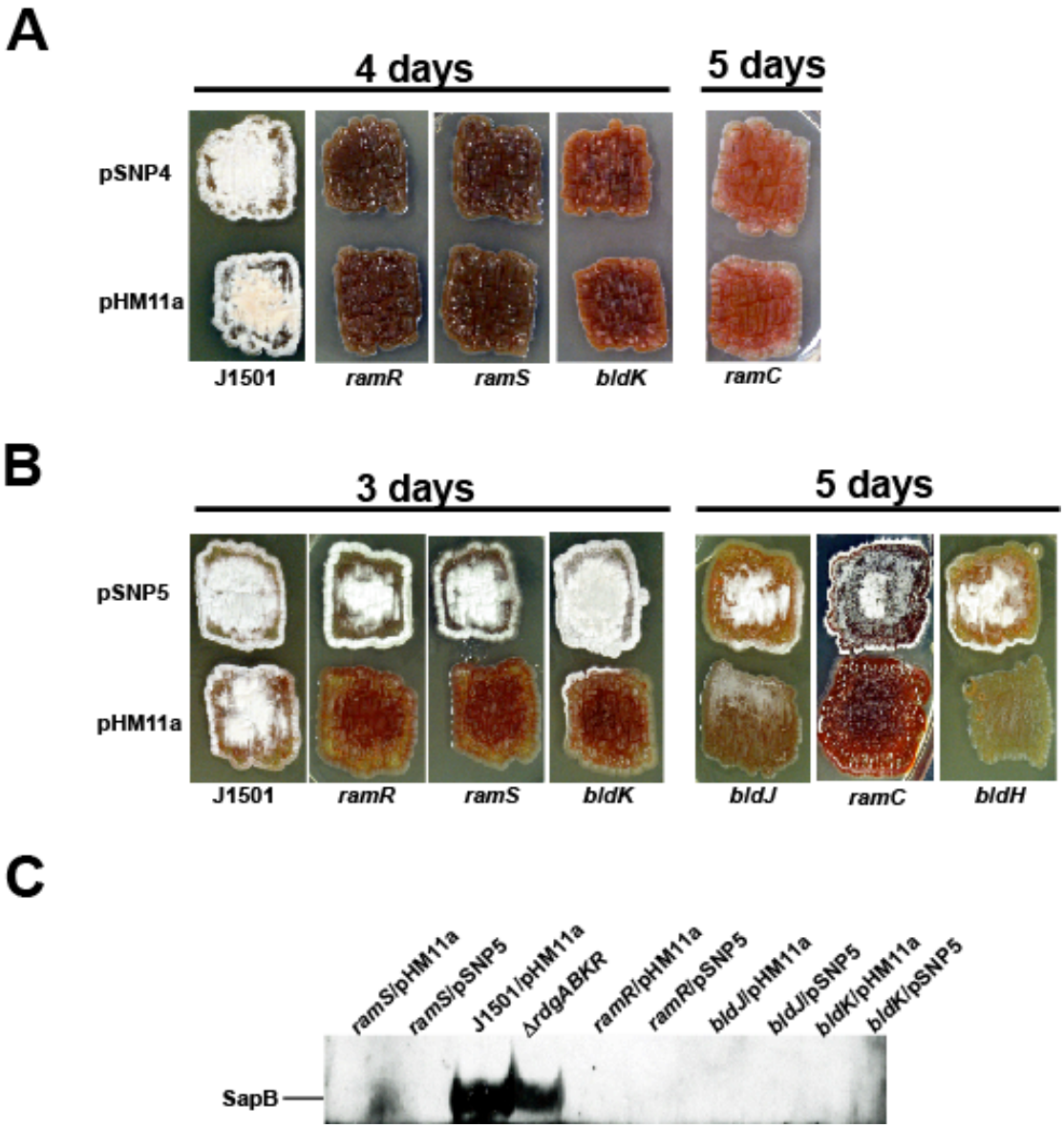

Fig. 12. SapB-independent rescue of *bld* and *ram* developmental mutants by *rdgR(D53E)* overexpression.
(A) The mutant D53N allele of *rdgR* was cloned into pHM11a (pSNP4) and introduced into either *S. coelicolor* J1501 or a series of *bld* and *ram* mutants. None of the developmental mutants overexpressing *rdgR(D53N)* (pSNP4) was rescued during development on R2YE plates.
(B) The D53E allele of *rdgR* was cloned into pHM11a (pSNP5) and introduced into either *S. coelicolor* J1501 or a series of *bld* and *ram* mutants. Suppression of the morphological defect by induction of AHF could be observed in all of the developmental mutants overexpressing *rdgR(D53E)* (pSNP5) during development on R2YE plates.
(C) Western blot analyses of SapB in mycelium- and spore-associated protein extracts from J1501, *ΔrdgABKR null* mutant and *bld-ram* mutants carrying either pHM11a or pSNP5 after 7 days of growth on R2YE agar plates. Extracts (15 μg) were separated by discontinuous polyacrylamide gel electrophoresis (see Experimental Procedures), transferred onto PVDF membranes, and probed with SapB antibodies.

Overexpression of *ramR* restores AHF in all *bld* mutants tested, in part through *ramS* transcription and SapB biosynthesis (Nguyen *et al.,* 2002). By analogy, developmental rescue of *bld* and *ram* mutants overexpressing *rdgR(D53E)* could be mediated in part by SapB. To address this question, immunoblots were

used to assay SapB in mycelial and spore extracts of both the *rdgR(D53E)* rescued *bld* and *ram* mutants (pSNP5) and the *ΔrdgABKR null* mutant strain. SapB was produced by the wild type strain, J1501 and, interestingly, also by the *ΔrdgABKR null* mutant strain (Fig. 12C), demonstrating that the deletion of the entire *rdgABKR* operon did not affect SapB biosynthesis. Furthermore, SapB was not detected (Fig. 12C; data not shown) in any of the RdgR(D53E)-rescued *bld* and *ram* mutants. These observations suggested that other signals downstream of *ramR* and *ramS*, but *rdgR* dependent, could be sufficient to activate a SapB independent mechanism of aerial hyphae formation and sporulation in *S.coelicolor*.

RdgR(D53E) overexpression restored AHF in *bld* mutants through extracellular complementation.

Extracellular complementation data have suggested a model where SapB is the final output of an extracellular signaling cascade inducing aerial hyphae formation (Willey *et al.,* 1993). All *bld* mutants are impaired in SapB production when grown separately. However, they may produce other morphogenetic compounds that trigger AHF or sporulation (Claessen *et al.,* 2003; Claessen *et al.,* 2004; Willey *et al.,* 2005). To test whether *bld* and *ram* mutant strains overexpressing *rdgR(D53E)* (pSNP5) could generate diffusible morphogenetic signal(s) able to elicit erection of aerial hyphae in recipient *bld* strains, extracellular complementation experiments were performed (Fig. 13). The morphogenetically blocked, *bldA*, *adpA* and *ramC* mutants were used as recipient strains and grown in close proximity to different combinations of RdgR(D53E)-rescued *bld* and *ram* mutants. While these cultures did not produce detectable SapB (Fig. 12C; immunoblots not shown), fringes of aerial hyphae, induced by extracellular complementation, were observed in each recipient strain only along the edges of the mycelium in close proximity to donor strains overexpressing *rdgR(D53E)* (Fig. 13; data not shown). This progressive phenotype, observed between five and ten days of incubation at 30°C, indicated that SapB independent diffusible signal(s) can promote AHF.

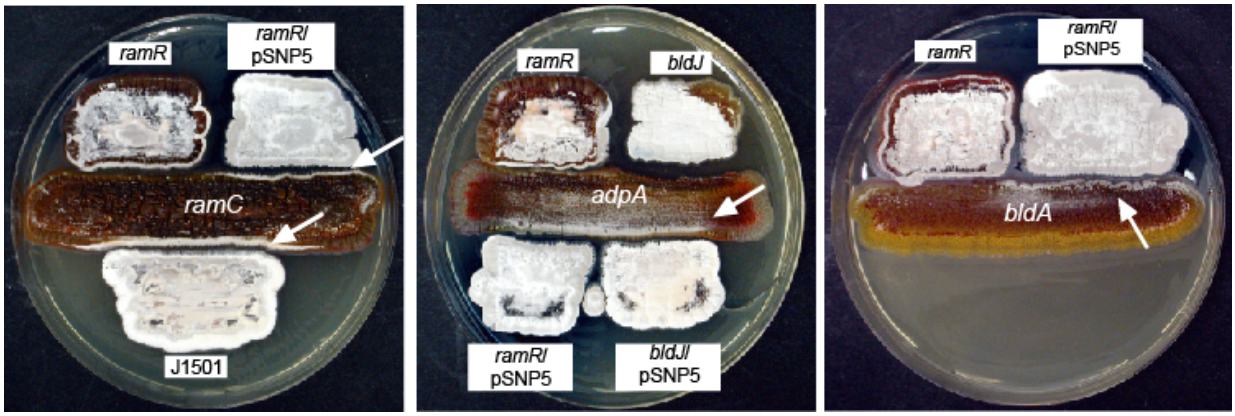

Fig. 13. Extracellular complementation of *bldA*, *adpA* and *ramC* mutants by bald strains overexpressing the D53E mutant allele of *rdgR* (pSNP5).
Extracellular complementation of recipient strains (*bldA*, *adpA*, *ramC*) was monitored over time every 12 h. White arrows indicate white fringes of aerial hyphae being formed along the edges of the mycelium placed in front of any *rdgR(D53E)* overexpressing strain (pSNP5).

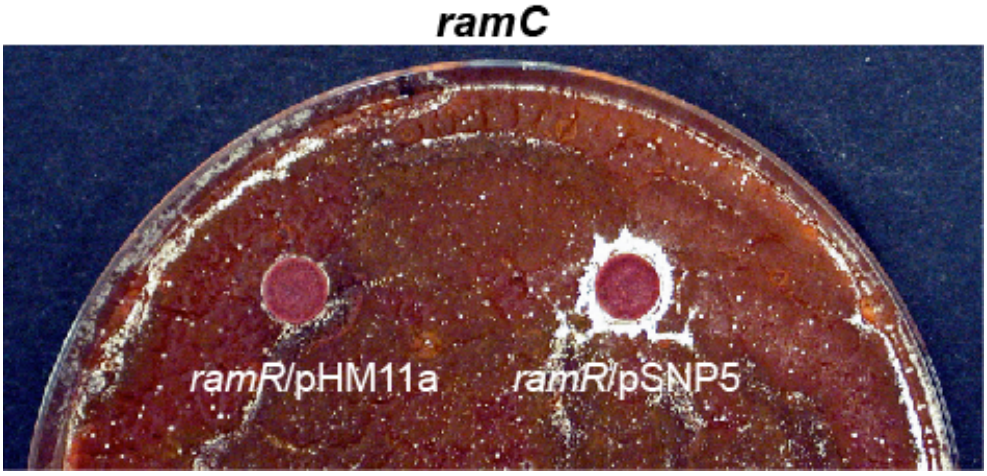

Fig. 14. Induction of AHF in the *ramC* mutant by application of total ethylacetate extracts of the *ramR* mutant overexpressing *rdgR(D53E)*.
Synchronously pregerminated spores of *ramR/pHM11a* and *ramR/rdgR(D53E)* (pSNP5) were inoculated on R2YE plates for 5 days at 30°C. Cultures were extracted with ethylacetate and total extracts were tested for their ability to induce AHF to the *ramC* mutant (see Experimental Procedures). Only the total extract from *ramR/rdgR(D53E)* was able to restore AHF to the *ramC* mutant after 4 days of incubation at 30°C. This is indicated by the white ring of aerial hyphae formed around the disk soaked with a saturating amount of *ramR/rdgR(D53E)* (pSNP5) extract.

RdgR(D53E) triggers the production of a hydrophobic morphogenetic compound(s).

To confirm that SapB independent signal(s) were produced upon *rdgR(D53E)* overexpression, we tested whether ethylacetate extracts of R2YE solid cultures of the *ramR* mutant overexpressing *rdgR(D53E)* (*ramR*/pSNP5; see Experimental Procedures) were able to induce AHF to the morphologically blocked *ramC* mutant. Similar extracts were prepared with solid R2YE cultures of the *ramR* mutant (*ramR*/pHM11a) grown on the same conditions and used as negative control. Unlike the extracts of the *ramR* mutant, application of the *ramR/rdgR(D53E)* extracts clearly induced AHF to a 3 days old R2YE culture of the *ramC* mutant after 4 days of incubation at 30°C (Fig. 14). A similar assay, performed with ethylacetate extracts of liquid cultures of the *ramR* mutant overexpressing *rdgR(D53E)* and the *ramR*/pHM11a mutant did not resulted in induction of AHF in the *ramC* mutant (data not shown). This strongly suggested that RdgR(D53E), directly or indirectly, may trigger the production of a hydrophobic morphogenetic compound(s) during development on solid R2YE medium that, similarly to SapB, could elicit AHF to the morphologically blocked *ramC* mutants (Fig. 14; data not shown).

***E. coli* cell extracts overexpressing the D53E form of RdgR can bind to the *rdgABKR* promoter upstream of the RamR binding sites.**

By analogy to other response regulators, RdgR may autoregulate its own transcription within the *rdgABKR* operon (Domian *et al.,* 1999; Stein *et al.,* 2002; Heermann *et al.,* 2003; Pragai *et al.,* 2004; Ainsa *et al.,* 1999). RamR binding sites are located within the first 80 bp upstream of the transcription initiation site on the 560 bp *rdgABKR* promoter region (Fig. 9A). As genetic studies had suggested that RdgR requires phosphorylation at the aspartate residue 53 to fulfil its *in vivo* function (Fig. 11; 12A, B), the D53E mutant allele of *rdgR* was cloned in *E. coli* strain Rosetta pLysis using an IPTG inducible promoter (pET11c) (see Experimental Procedures). EMSA were carried out using increasing amounts of total cell extracts of *E. coli* expressing RdgR(D53E) (Fig. 15A) to probe the 180 bp (small) or the 380 bp (large) *Hin*fI fragment of the *rdgABKR* promoter region (SF* and LF* probes in Fig. 7A; Fig. 15B, C). Both IPTG-induced and uninduced extracts of *E. coli* cells containing pET11c were used as negative controls. As shown in Fig. 15B, the RdgR(D53E) containing cell extracts, but not the control extracts, caused a discrete shift in the mobility of the LF* probe. None of the extracts altered the mobility of the SF* probe (Fig. 15C), where the RamR binding sites were mapped (Fig. 7A, C; 9A). These results indicated that RdgR(D53E) could bind to an operator site(s) located on the 380 bp *Hin*fI fragment of the

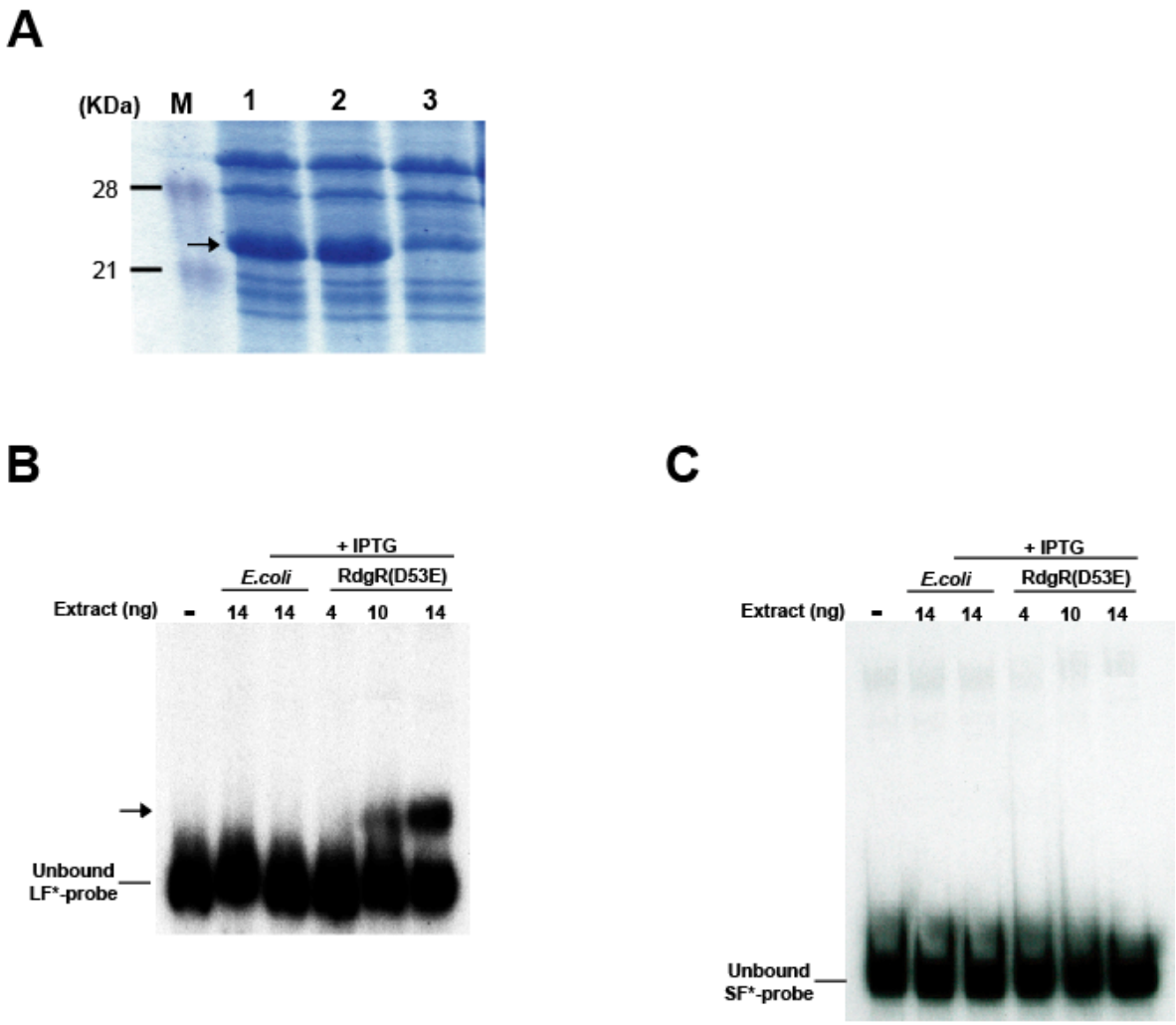

Fig. 15. Specific binding of RdgR(D53E) to the *rdgABKR* promoter region.
(A) Cell extracts of IPTG-induced *E. coli* strain Rosetta pLysis containing the control plasmid pET11c (lane 3) and *E. coli* strain Rosetta pLysis expressing RdgR(D53E) (lanes 1, 2): 15 µg of total protein extracts were separated in a 15% polyacrylamide gel and stained with Coomassie blue. M corresponds to the protein molecular weight marker and the black arrow indicates the IPTG-induced RdgR(D53E).
Increasing amounts of RdgR(D53E) containing *E. coli* cell extracts were incubated with either the 380 bp *Hinf*I LF* (B) or the 180 bp *Hinf*I SF* DNA-fragments (C and Fig. 7A). IPTG-induced and uninduced *E. coli* cell extracts (14 ng), harbouring the plasmid pET11c, were used as controls. The black arrow indicates the shift in the mobility of the LF*-probe (B) upon incubation with *E. coli* cell extracts expressing RdgR(D53E). No shift in the mobility of the SF*-probe was detected (C).

rdgABKR promoter (Fig. 7A; 15B), upstream of the RamR binding sites. When the same experiments were carried out with *E. coli* cell extracts expressing wild type RdgR or RdgR(D53N) protein, no shift in the mobility of the LF* probe was observed, further supporting the prediction that RdgR requires phosphorylation to be fully active in binding to its operator sites (data not shown).

Discussion

Discovery of RamR-dependent developmental genes (*rdg*) located outside of the *ram* locus

Colonial morphogenesis of *S. coelicolor* is programmed by a complex interplay of regulatory cascades having metabolic, morphological, homeostatic and stress-related checkpoints (for a review see Chater, 2001). The response regulator, RamR, may represent a final checkpoint that commits the colony to activate the sporulation program only if physiological imbalances, such as those represented by the *bld* genes, are not encountered (Nguyen *et al.,* 2002). RamR exerts part of its key regulatory function through direct transcriptional activation of the adjacent SapB biosynthetic genes, *ramC* and *ramS* (Nguyen *et al.,* 2002) (Kodani *et al.,* 2004).

However, unlike *ramR* overexpression, co-expression of *ramS* and *ramC* did not allow aerial hyphae formation in the *citA* or the *bldJ* mutants (our unpublished data; Nguyen *et al.,* 2002). This suggested that RamR activated other essential developmental genes that are located outside of the *ram* cluster. Furthermore, the ability of the *ramR* mutant to sporulate later, in the absence of SapB, suggested that such genes may be part of a secondary, delayed-onset, developmental pathway.

To identify these genes and record gene expression that underlies this alternative program, DNA microarray and quantitative real time RT-PCR analyses were performed, comparing the expression profiles of J1501 ($SapB^{+}$), *ramR* mutant ($SapB^{-}$) and J1501 overexpressing *ramR* ($RamR^{++}$, $SapB^{++}$). Growth was not affected by *ramR* inactivation or overexpression and gene transcription profiles were comparable in the three strains. This facilitated the identification of two related clusters of *ramR* dependent genes, including not only the previously known members of the regulon, *ramCSAB*, but also four uncharacterized genes, *rdgABKR,* that formed an operon (Fig. 6A, B). Furthermore, *rdg* genes were switched on during development, with transcriptional profiles that were similar to the *ramCSAB* genes (Fig. 2; Fig 4).

Transcription of *rdgABKR* was driven by RamR binding, as confirmed by EMSA and DNaseI footprinting studies of the *sco4075* (*rdgA*) promoter using recombinant unphosphorylated RamR (Fig. 6C; Fig. 7). While RamR probably requires phosphorylation by unidentified histidine kinase(s) to be fully active (O'Conner *et al.,* 2005; Nguyen *et al.,* 2002) it can form a stable dimer *in vitro* and cooperatively bind to its operator sites on the *ramC* promoter (O'Conner *et al.,* 2005). Our results showed that unphosphorylated RamR also bound to three sites on the *rdgA* promoter, with a binding coefficient (Kd

estimation= 8 x 10^{-8} M) similar to that reported for the *ramC* promoter (Nguyen *et al.,* 2002; O'Conner *et al.,* 2005).

RamR binds to operator sites of about 12 bp in its target promoters which define its consensus recognition sequence TGTC(A/C)(G/C)RNYC. Although the predicted RNAP hexameric recognition sequences of *ramC* and the *rdgA* promoters (centred at the -10 and -35 regions) do not share any sequence homology, RamR operator sites have similar arrangements. Two strong binding sites are localized upstream (about 10 bp) and one weak binding site overlaps with the predicted -35 region (Fig. 9). In addition, while bound to the *rdgA* promoter, RamR altered DNA conformation, as indicated by the formation of DNaseI hypersensitive sites that flank the protected regions (Fig. 7C). The following sections propose a model in which RamR responds to a developmental signaling regulatory cascade by activating transcription of the *ramCSAB* and *rdgABKR* operons (Fig. 16). This elicits a rapid switch to aerial hyhae formation, dependent on SapB. When the SapB dependent pathway is inactivated in the *ramS* mutant, an *rdg*-dependent pathway involving other morphogenetic compounds can also activate aerial hyphae formation. Later *rdgABKR* plays a role in morphogenesis of aerial hyphae into spores (Fig. 16).

The *rdgABKR* operon and the commitment of aerial hyphae to sporulation

To determine whether the *rdgABKR* operon could play roles in development, we constructed a *ΔrdgABKR* null mutant by deleting the entire gene locus. Unlike *ramR*, *ramS* (both delayed in AHF), and *ramC* (defective in AHF) mutants (Nguyen *et al.,* 2002), the *ΔrdgABKR null* mutant was able to synthesize SapB and erect aerial hyphae, showing that *rdgABKR* is dispensable for SapB biosynthesis and AHF (Fig. 12C; Fig. 16). Nevertheless, the *ΔrdgABKR* null mutant produced defective aerial hyphae and was severely delayed in converting them to spores on R2YE medium (Fig. 10). Aerial hyphae produced by the *ΔrdgABKR* mutant were impaired in the two initial phases of sporulation, i.e. curling, and septa formation.

Earlier studies have identified a group of sporulation (*whi*) mutants (Chater, 1972; Kelemen *et al.,* 1996; Kelemen and Buttner*,* 1998; Ryding *et al.,* 1998; Ryding *et al.,* 1999; Flärdh *et al.,* 1999), which genetically defined the successive steps of aerial hyphae differentiation leading from growth arrest, coiling, and spore septation, to grey pigment formation in morphologically mature spores. Thus, mutants that were able to produce grey spores were systematically discarded in the screen for white (*whi*)

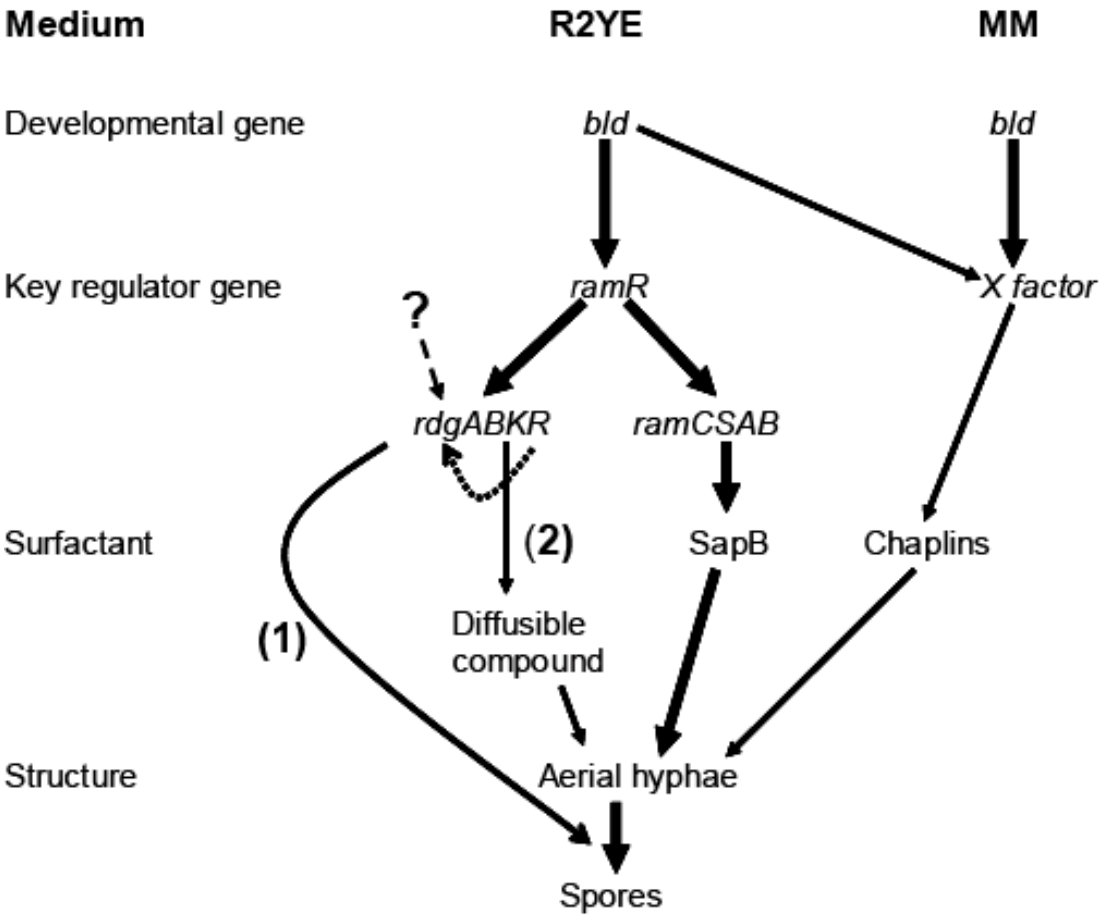

Fig. 16. Model for the central regulatory role of RamR regulon in the initiation of the morphological differentiation of *S. coelicolor*.
RamR is a master response regulator that integrates *bld* gene-dependent signals when development occurs on complex media. Upon binding to the promoter regions of the *ramCSAB* and *rdgABKR* operons, RamR activates and coordinates their transcription, triggering pathways that will induce AHF and commit them to sporulation. On complex medium, SapB, generated by post-translational modifications of its precursor peptide RamS, represents the main pathway for erection of aerial hyphae. A second pathway is mediated by the products of the *rdgABKR* operon. This may function by triggering transduction events that promote the commitment of the emerging aerial hyphae to sporulation (1) and secretion of a yet uncharacterized diffusible compound that, like SapB, can elicit aerial growth (2). The ability of RdgR, then, to bind to the *rdgABKR* promoter region (dotted arrow), upstream of RamR binding sites, suggests the existence of an autoregulatory loop that may maintain expression of these genes after induction by RamR or other unidentified signals (question mark). In addition to SapB and the products of the *rdgABKR* operon, another group of morphogenetic proteins, the Chaplins, is required for the erection of aerial hyphae on both minimal and rich medium. Expression of *chp* genes is *bld* dependent but RamR independent and, in analogy with the RamR pathway, is probably triggered by an unidentified master regulator X.

colonies. However, unlike the classical *whi* mutants, the *ΔrdgABKR* mutant formed aerial hyphae that were unusually branched, developing some gray pigment and only a few regular spore chains after a delay of two days (Fig. 10). Long straight and undifferentiated aerial hyphae with occasional branches, similar to those of the *ΔrdgABKR* mutant, were reported only in the *whiG* mutant (Chater, 1972; Flärdh *et al.,* 1999). The *whiG* gene product encodes an RNA polymerase σ factor, whose activation (after release from an anti-σ factor; Kelemen *et al.,* 1996) represents an "early" developmental check-point that commits aerial hyphae to the sporulation pathway. This developmental switch is preceded by a change from vegetative-type growth, characterized by branching, to aerial-type unbranched mycelium specialized for sporulation (Flärdh *et al.,* 1999). The morphological similarities between the *whiG* and the *ΔrdgABKR* mutants, suggest that the *rdgABKR* operon might commit aerial hyphae to sporulate through the activation of the WhiG signaling cascade (pathway 1 in Fig. 16). Interestingly, unlike SapB-rescued *bld* mutants (Tillotson *et al.,* 1998), where aerial hyphae are unable to differentiate into spores, extracellular application of purified SapB (our unpublished data; Kodani *et al.,* 2005) or other biosurfactants such as SapT and SC3 to *ramS* mutant leads to the erection of aerial hyphae that undergo normal steps of sporulation (Kodani *et al.,* 2005; our unpublished data). This implies that, in the *ramS* mutant (but not in *bld* mutants), where *ramR* and *rdgABKR* are expressed (our data not shown), regulatory events that lead to sporulation may still be activated once aerial hyphae have breached the air-water interface, independently from SapB. We propose that the *rdgABKR* operon might function as part of this transduction network and that its transcriptional dependence on RamR would couple the onset of AHF elicited by SapB biosynthesis with the initiation of the sporulation program (Fig. 16).

Complete morphological differentiation is induced by expression of constitutively active RdgR in several developmental mutants

The *rdgABKR* operon encodes two putative subunits of an ABC transporter (*rdgA* and *rdgB*), a histidine kinase (*rdgK*) and a response regulator (*rdgR*) (Fig. 6A). To elicit aerial growth in bald mutants, RdgR apparently requires phosphorylation of its aspartate residue at position 53; the RdgR(D53N) mutant allele had no activity *in vivo* (Fig. 12A, B). The ability of *rdgK* and *rdgR* (co-expressed by pSNP3) or the constitutively active *rdgR(D53E)* mutant allele to restore AHF and sporulation to all developmental mutants tested, demonstrated that expression and activation of RdgK and RdgR is sufficient to elicit

morphological differentiation utilizing a pathway which acts downstream of RamR and SapB (Fig. 11; 12).

RdgR and RdgK apparently form a two component signal transduction system activated by an environmental signal. It is interesting to speculate that an externally exposed sensor RdgK phosphorylates its cognate response regulator, RdgR, in response to the radically different environments encountered by the hyphae as they emerge from liquid into the air. The D53E form of RdgR, a mimic of the activated phosphoprotein, bound to an operator site(s) upstream of the RamR binding sites in the *rdgABKR* promoter region (Fig. 15). Thus, *rdgABKR* transcription may be activated initially by RamR but its activity is modulated by a second input elicited by environmental signals that activate RdgK to phosphorylate RdgR. In principle, phosphorylated RdgR may act as a repressor to decrease transcription of the operon. However, its binding site suggests that it acts in a positive auto regulatory loop that may underlie its role as a genetic switch. Irreversible developmental switches are often based on regulatory proteins that are able to amplify and thus stabilize their own activity. Thus, under the conditions we tested, once RamR has activated the transcription of the *rdgABKR* operon and RdgR is phosphorylated by environmental signals, RdgK and RdgR expression may initiate an autoregulatory loop (Fig. 16) supporting an irreversible switch that forces the mycelium down the sporulation pathway. Upon phosphorylation, RdgR may activate transcription of downstream genes (Fig. 16).

In addition to the commitment to sporulation (pathway 1 in Fig. 16), RdgR may contribute to AHF by activating production of an extracellular signaling compound(s). This may act independently of SapB (Fig. 11; 12; 14; immunoblots not shown), slowly diffusing through the medium and rescuing recipient *bld* mutants (pathway 2 in Fig. 16; Fig. 14). Other compounds produced by other *Streptomyces* species stimulate aerial growth. Examples are the γ-butyrolactone A-factor in *Streptomyces griseus* and the antibiotic pamamycin in *Streptomyces alboniger* (Khokhlov *et al.,* 1973; McCann and Pogell 1979; Beppu 1992). The lantibiotic SapT from *Streptomyces tendae*, perhaps also having a functional homolog in *S. coelicolor*, activates AHF in SapB deficient mutant strains (*bldJ* and *ramS* mutants (Kodani *et al.,* 2005). In addition to SapB, *S. coelicolor* produces another group of morphogenetic proteins, the chaplins, which are anchored to the cell wall of the mycelium, where they act as biosurfactants for the erection of aerial hyphae on both minimal and rich medium. Chaplins represent another class of hydrophobic secreted proteins required for morphological differentiation in *S. coelicolor* (Claessen *et al.,* 2003; Claessen *et al.,* 2004; Elliot *et al.,* 2003) (Fig. 16). Some Chaplins, such as Chaplin E and H, as well as the

morphogenetic lantibiotic SapB, can be found in the medium of both liquid and solid cultures (our data not shown and Nguyen *et al.,* 2002), suggesting that they can slowly diffuse from the surface of the embedded mycelium while they are assembling at the water-air interface (Claessen *et al.,* 2003; Claessen *et al.,* 2004). Similar to SapB, the predicted morphogenetic compound produced upon RdgR activation may be exported to the surface of the vegetative mycelium via an ABC transporter (probably formed by RdgA and RdgB) and elicit initiation of aerial growth in combination with SapB and Chaplins (pathway 2 in Fig. 16). Chemical purification and structural determination of the RdgR-dependent morphogenetic compound will clarify whether any regulatory interconnection may exist among the SapB, the *rdgABKR* and the chaplins pathways during morphological differentiation in *S. coelicolor*.

Thus in addition to activating a SapB-dependent system for AHF, RamR exerts a central role in development of *S. coelicolor* by activating transcription of another group of previously unidentified morphogenetic genes (*rdgABKR*). These genes provide functions needed for both aerial hyphae formation and sporulation, demonstrating integrated circuits connecting these sequential developmental processes.

Experimental Procedures

Strains and growth conditions

E. coli strain XL1 Blue, used to propagate all plasmids, was grown in liquid or solid LB medium at 37°C. *E. coli* strain Rosetta pLysis (Novagen) was used to express recombinant proteins in liquid LB cultures at 30°C. *S. coelicolor* strains were grown at 30°C in R2YE, MS, YEME (Kieser *et al*., 2000) or J media (Puglia *et al*., 1995).

The *Streptomyces* strains used in this study were: *S.coelicolor* J1501, *ramR* mutant (BZ5; Nguyen *et al*., 2002); *S.coelicolor* J1501 overexpressing RamR (J1501/ *ramR*) (pKN22; Nguyen *et al*., 2002); *ramS* mutant (Nguyen *et al*., 2002); *ramC* mutant (Nguyen *et al*., 2002); *adpA* mutant (Nguyen *et al*., 2003) as well as *bld* mutants (*bld261*, *bldK*, *bldA*, *bldH*) (Willey *et al.,* 1993; Nodwell *et al.,* 1996).

Transformation and conjugation

E. coli transformants were selected with ampicillin (100 μg ml^{-1}), hygromycin (100 μg ml^{-1}) or kanamycin (50 μg ml^{-1}). To avoid restriction of methylated DNA, the *dam*$^{-}$*dcm*$^{-}$ *E.coli* strain ET12567 (MacNeil *et al*., 1988) was used for conjugation with *S. coelicolor* and to prepare plasmids used to transform *S. coelicolor* protoplasts (Kieser *et al.* (2000). Ex-conjugants were selected by overlaying plates with aqueous solutions (1 ml) of hygromycin (2 mg/ml) and *S. coelicolor* transformants with aqueous solutions of thiostrepton (0.5 mg/ml).

PCR amplification

All polymerase chain reaction (PCR) amplifications were done using a GC-RICH kit (Roche) and cloned into pGEMT-easy (Promega). Correct sequences were routinely verified.

Overexpression of RdgK, RdgR, RdgKR, RdgR(D53E) and RdgR(D53N) in *S. coelicolor* strains

To express *rdgK* from the constitutive *ermE* promoter, the coding region was PCR amplified from *S. coelicolor* 1501 genomic DNA using primers NdeI-*rdgK* (5'-CATATGCGCAGGCCGGGCGGATG-3') and *rdgK*-BamHI (5'-GGATCCCGCACGGTCGTCGTCACGG) (restriction sites were underlined). The insert was cloned in pGEMT-easy vector (pGEK), digested with *Nde*I- *Bam*HI and ligated into the same sites on pHM11a (pSNP1) (Motamedi *et al.,* 1995). To express *rdgR* from the *ermE* promoter, the coding

region was PCR amplified using primers NdeI-*rdgR* (5'-CATATGGTGCGCGTTCTGCTCGCCGA-3') and *rdgR*-BamHI (5'-GGATCCCTATACCCAACCGCGCTCC-3'). The insert was cloned into the pGEMT-Easy vector (pGER), digested with *Nde*I- *Bam*HI and ligated into the same sites on pHM11a (pSNP2). To express *rdgR* and *rdgK* from the *ermE* promoter, the coding region was PCR amplified with primers NdeI-*rdgK* and *rdgR*-BamHI. The fragment was excised from pGEMT-easy vector (pGEKR) by *Nde*I and *Bam*HI digestion and ligated into the same restriction sites in pHM11a (pSNP3).

The D53E and D53N mutant alleles of *rdgR* were generated as described below. Corresponding plasmids, (pSNP4 and pSNP5) were used for constitutive expression of RdgR(D53E) and RdgR(D53N) in *S. coelicolor*.

Construction of the *rdgABKR* deletion mutant in *S. coelicolor* J1501

Primer sets (restriction sites were underlined) *Hind*III -Δabkr1 (5'-AAGCTTGCTGTACCGGAACGAGGCGGGCGA-3') and Δabkr5 (5'-GCGAGGGAGCGCGGTTGGGTATAGTG-3') or Δabkr3ht (5'-CTATACCCAACCGCGCTCCCTCGCCACGTGTTCGTGCTCGTTCGTTTGCAT-3') and Δabkr4 (5'-CGGGCAGATTTAGTCGGTCTTCCCATT -3') were used to PCR amplify two 1 Kb fragments (Δabkr-down and Δabkr-up) downstream and upstream of the *rdgABKR* locus. These two PCR fragments included 24 bp of overlapping sequence.

The Δabkr-down and Δabkr-up PCR products were purified using a QIAquick PCR purification kit (Qiagen), and 5 ng of each were used as template in the same reaction mix for a second PCR amplification with primers *Hind*III -Δabkr1 and Δabkr4 to generate the 2 kb fragment, Δabkr, in which the *rdgABKR* genes were deleted. The fragment was cloned into pGEMT-easy (pGEΔ), then excised with *Hind*III and *Eco*RI and ligated into pSET151 at the same restriction sites (pSEΔ). A kanamycin cassette (Reece *et al*., 1995) on a *Sph*I fragment was introduced into pSEΔ at the *Sph*I site within the Δabkr fragment, its orientation was confirmed, and the resulting plasmid, pSKΔ, was used to transform J1501 protoplasts. Three kanamycin resistant and thiostrepton sensitive *rdgABKR* null mutant candidates were isolated from independent transformants after two rounds of growth in YEME under non selective conditions. Replacement of the *rdgABKR* genes with the kanamycin cassette in the 3 *rdgABKR null* mutant candidates was confirmed by PCR with primers DEL-down (5'-GGCGCGACAGCCACTATACCCAAC-3') + DEL-up (5'-GATGACGGCTCGCACTGTCGTCC-3'); NdeI-

rdgK + *rdgR*-BamHI; NdeI-*rdgK* + *rdgK*-BamHI; *rdgA* (5'-ATTGAGGTCACCGACCTTCGG-3') + *rdgR*-BamHI.

Complementation of the *rdgABKR null* mutant

The primer pair (restriction sites were underlined) NdeI-com/*rdgA* (5'-CATATGATTGAGGTCACCGACCTTCGG-3') and *rdgR*-BamHI was used to PCR amplify the coding regions of the *rdgABKR* operon from genomic DNA of *S. coelicolor* J1501. The insert was cloned in pGEMT-easy vector (pGERDG), digested with *Nde*I- *Bam*HI and ligated into the same sites on pHM11a (pSNP6). This plasmid, pSNP6, was then used to complement the *rdgABKR null* mutant of *S. coelicolor*.

SapB detection

SapB was extracted from the surface of mycelium as described by Guijarro *et al*., 1988 and partially purified as described by Willey *et al.,* (1991). Protein extracts were resolved by discontinuous polyacrylamide gel electrophoresis (Schägger and von Jagow (1987) and SapB was visualized using the ECL Plus Western blotting detection system (Amersham Pharmacia; Willey *et al.,* 1991).

RNA isolation

For RNA isolation, fresh spores of *S. coelicolor* J1501, *ramR* mutant and J1501/ *ramR* overexpressing strains were pregerminated (Kieser *et al*., 2000) and inoculated onto cellophane discs on the surface of R2YE medium. Mycelium was scraped from the cellophane discs at different times during development and immediately treated with RNAprotect bacteria reagent (Qiagen) for 15 min to stabilize the *in vivo* transcript profile. Mycelia were centrifuged at 5,000 x g for 10 min at RT. The pellet was resuspended in 5 ml of RLT^{TM} buffer (Qiagen), sonicated for 15 sec (3x with an output of 80W), and centrifuged at 12,000 x g for 15 min at 4°C to remove cell debris. The supernatant was extracted in phenol/chloroform and then chloroform. The aqueous phase was added to 3 ml of ethanol and loaded onto Rneasy Midi columns (Qiagen). The final steps of RNA purification were carried out according to the protocol in the Rneasy Midi kit (Qiagen). DNA contamination was removed from the RNA samples by DNA-*free*^{TM} kit (Ambion). The quality of the RNA samples was verified by RNA 6000 Nano Assay kit

(Agilent Technologies) and by agarose gel electrophoresis. Quantification of RNA was carried out by adsorption at 260 nm.

Microarray analysis and quantitative real time RT-PCR

Microarray analysis of RNA samples was performed as described by Huang *et al*., 2001. For quantitative real time PCR analysis, first-strand cDNA synthesis was carried out using 2 µg total RNA and SuperScript II (Invitrogen), following the manufacturer's instructions (Cat. No.18064-014). Quantitative real-time PCR of selected genes was performed using the Bio-Rad iCycler™ Real-Time PCR Detection System and iQ™ SYBR Green Supermix Kit (170-8880). The first-strand reaction (5%) was used as DNA template. Real-time PCR conditions were as follows: 94°C for 10 min, 40 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. The amount of target cDNA was determined by standard curves and normalized internally to 16S rDNA levels. Transcript abundance was calculated as $\log_2$ of normalized values. Relative transcript abundance was calculated as $\log_2$ of normalized ratios. (See Table 1 for all quantitative real time RT-PCR primers).

S1 nuclease mapping

For S1 nuclease mapping of *rdgA*, 45 µg of RNA was hybridized to a γ–[^{32}P] end-labeled probe at 45°C for 4-15 h following denaturation at 65°C for 15 min. S1 nuclease (Roche) digestions were performed as described by Kieser *et al*. (2000) and digestion products were separated on an 8% sequencing gel. Probes having a unique end label were generated by 5' end labelling of prdg-S1 primer (5'-GCAGGGCGAAGACCTCACCGCGG-3'), internal to the *rdgA* coding sequence, using γ–[^{32}P] ATP and T4 polynucleotide kinase (Biolabs). Labeled primer (30 µM) was used together with unlabeled universal primer (15 µM) in a PCR reaction generating a fragment of 300 bp. The 400 bp *hrdB* probe and the 300 bp 16S *rrnA* probe, used as positive controls for RNA integrity and loading, were prepared using either the radiolabeled hrdB-S1 primer (5'-GCCATGACAGAGACGGACTCGGCG-3') or the 16S-S1 primer (5'-GCTTGTCCCAGAGTGAAGGGCAG-3') together with unlabeled universal primer in a PCR reaction. Probes contained non-homologous 3' extension (about 50 nucleotides) to discriminate between full-length protection and probe-probe reannealing products. Sequencing ladders were produced using the T7 sequencing kit (Amersham Pharmacia Biotech) with the *rdgA* internal primer.

Tab 1. Primer pair used for the real time PCR amplification of specific gene targets

Primers	**Sequence**
qRT-rdgR-Fw	5'-GTGCACGCCGGAAACCGTTA-3'
qRT-rdgR-Rv	5'-GCGGCCGACGACAGGTAGTT-3'
qRT-rdgK-Fw	5'-CTCGACGACGCCAGGGAGAC-3'
qRT-rdgK-Rv	5'-GATGCGCTGCACCTCGATCA-3'
qRT-rdgB-Fw	5'-CTCGCCTCCGGCCTGTTCTT-3'
qRT-rdgB-Rv	5'-GCCGCCGTACAGCAAACACC-3'
qRT-rdgA-Fw	5'-AACTGGGCGTCAGCGACCA-3'
qRT-rdgA-Rv	5'-CTGCTTGGCGATGCTGAGGA'3'
qRT-ramC-Fw	5'-CCGCACATCCTGAGCGACCT-3'
qRT-ramC-Rv	5'-ACTCCGGGATGCGGAAGACC-3'
qRT-ramS-Fw	5'-CAGTCGATGGAGACCCCCAAG-3'
qRT-ramS-Rv	5'-GGTGGTGATGCTCAGGCTGCT-3'
qRT-ramA-Fw	5'-CAGCGCACCCAGTCCCTGAT-3'
qRT-ramA-Rv	5'-CAGCAGCGGGACCAGGACAC-3'
qRT-ramB-Fw	5'-TGGGCGCGTTCACCTACCTC-3'
qRT-ramB-Rv	5'-TGACGCTGGCTCTGGCTCTG-3'
qRT-ramR-Fw	5'-TCACCGTGGCCCTGCTGAAG-3'
qRT-ramR-Rv	5'-GACCGCCGTGGCCATGTAGT-3'

RT-PCR analyses.

The Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to synthesize first-strand cDNA from purified DNA-free RNA preparations. For each cDNA synthesis reaction, 2 μg of RNA was denatured together with 50 ng of 72% GC-rich random hexamers, 10 mM dATP, 10 mM dTTP, 10 mM dCTP, and 10 mM dGTP (total volume of 10 μl) at 75°C for 5 min and quick chilled on ice. After collecting the contents of each tube by brief centrifugation, 10 μl of cDNA synthesis mix (2 μl of 10X RT buffer, 4 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT, 1 μl of 40 U/ μl of RNaseOUT™ , 1 μl of 200 U/ μl of SuperScript III RT) was added to each RNA/primer mixture to give a total volume of 20 μl. The reaction mixes were incubated at 25°C for 10 min, followed by 50 min at 50°C. The reactions were stopped at 85°C for 5 min and chilled on ice. RNA was removed by adding 1 μl (2 U) of RNase H at 37°C for 20 min. PCR reactions included 15 μM of each primer and 2 μl of cDNA. The PCR programme was as follows: 30 sec at 96°C (denaturation), 10 sec at 64°C (annealing) and 25 sec at 72°C (elongation) for 25 cycles. The reaction was completed by incubating for 7 min at 72°C. Samples were separated on 2% agarose gels in TBE buffer and stained with ethidium bromide. The following combinations of primers were used: rdg-coAB (5'-GTGGAGCTGCGCCGCCTGGA-3') and rdg-coBA (5'-GCTGAACGGCATCACGAACGG-3') for *rdgA* and *rdgB* cotranscription; rdg-coBK (5'-TGTGCGAGCTGCTGCCGCTG-3') and rdg-coKB (5'-CCAGCGCGAGGATCTCGATGA-3') for *rdgB* and *rdgK* cotranscription; rdg-coKR (5'-CTCTCGGCGGTGGACGGGAC-3') and rdg-coRK (5'-GATGTGGCGACCTTCACACCGT-3') for *rdgR* and *rdgK* cotranscription; hrdB-RT-Fw (5'-CAGATTCCGGCCACTCAGTGGAAGA-3') and hrdB-RT-Rv (5'-CGCCGTCGCCTTCTTGGCGGTCGT-3') for *hrdB.* RT-PCR experiments without prior reverse transcription were performed on all RNA samples to assure exclusion of contaminating DNA.

Preparation of DNA probes for gel mobility shift assays and DnaseI footprinting

Primers rdgFL-down (5'-TGTTCGTGCTCGTTCGTTTGCATGCT-3') and rdgFL-up (5'-CGTTCGCCGAGGCGCGGTGAGC-3') were used to PCR amplify and radiolabel (α-[^{32}P] dATP) a 560 bp fragment corresponding to the annotated intergenic region upstream of *rdgA*, encoding the promoter of the *rdgABKR* operon (FL-probe). To determine the minimal *rdgABKR* promoter fragment that could be bound by recombinant RamR, the FL-probe was purified by the QIAquick PCR purification kit (Qiagen)

and digested with *HinfI*, generating the smaller, 180 bp *Hinf*I fragment and the longer, 380 bp *Hinf*I fragment probes (SF and LF probes). After heat inactivation of the enzyme at 75°C for 15 min, 0.06 pmol of the *HinfI* digested promoter probe mix was incubated with 100 nM of $(His)_6$-tagged RamR protein (Nguyen *et al.,* 2002).

The end-terminally radiolabeled probes corresponding to the small *Hinf*I fragment (SF*-probe) and the large *Hinf*I fragment (LF*-probe) were generated with primers (restriction sites are underlined) rdg184SF-H (5'-AAGCTTGTTCGAGCCTCGTCGTCCGCTC-3') and rdgFL-down or with primers rdg380LF-H (AAGCTTGAGCGGACGACGAGGCTCGAAC) and rdgFL-up respectively. Fragments were cloned into pGEMT-Easy, removed by *Sph*I /*Hind*III digestion and ligated into pUC18 at the same restriction sites. Inserts were excised with *Sma*I and *Hind*III, gel purified and labeled with α-[^{32}P] dATP by fill-in reactions of the *Hind*III overhang using Klenow polymerase.

Electrophoretic mobility shift assay (EMSA).

EMSA was performed using the $(His)_6$-tagged RamR protein or increasing amounts of crude extracts of *E. coli* overexpressing RdgR(D53E) in 20 μl of TA buffer (10 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 100 mM NaCl, 2 mM DTT, 0.1% (v/v) TritonX-100, 10% glycerol) and 0.06 pmol of [^{32}P]-labeled DNA probe. poly-(dI-dC) (2 μg) was added to minimize non-specific protein binding. BSA (100 μg ml^{-1}) was added in EMSA reactions with $(His)_6$-tagged RamR protein. Reaction mixes were incubated at 30°C for 10 min and specific DNA-protein complexes were separated from free probe on a 5% non-denaturating acrylamide gel and visualized using a Phosphor Imager.

Dnase I footprinting assays

Dnase I footprinting analyses were performed using the same reaction conditions described for EMSA. Dnase I (Roche), diluted in Dnase I buffer (10 mM Tris/HCl pH 7.8, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM DTT, 100 mM NaCl and 10% glycerol) to 0.025 U μl^{-1}, was added to the samples (1 μl per reaction tube). The reaction was stopped by adding TE buffer (10 mM Tris/HCl pH 7.8, 50 mM EDTA), and then extracted with phenol-chloroform and chloroform. The samples were precipitated with ethanol and 1/10 volume of 3 M sodium acetate, resuspended in 14 μl of stop solution (Amersham), and resolved on a 8% polyacrylamide sequencing gel. Products of the reaction were visualized using a Phosphor Imager.

Experimental Procedures Chapter I

Site-directed mutagenesis of RdgR

Mutant alleles of *rdgR* in which aspartate 53 (D53) was replaced by glutamate (D53E) or by glutamine (D53N), were generated by a two-stage PCR procedure. Primers (mutagenized site is underlined) *Nde*I-*rdgR* and *rdgR*-BamHI were used together with either primer pair D53E-Rv (5'-GCCGGGCATCTGGAGCTCCAGTACGGCGAC-3') and D53E-Fw (5'-GTCGCCGTACTGGAGCTCCAGATGCCCGGC-3') or primer pair D53N-Rv (5'-GCCGGGCATCTGGAGCTGCAGTACGGCGAC-3') and D53N-Fw (5'-GTCGCCGTACTGCAGCTCCAGATGCCCGGC-3') to amplify the D53E and D53N alleles respectively. The fragments were cloned in pGEMT-easy (pGE-D53E and pGE-D53N), excised with *Nde*I- *Bam*HI, and ligated into the same sites on pHM11a to generate pSNP4 or pSNP5.

Construction of a *rdgR(D53E) E. coli* expression plasmid

pGE-D53E was digested with *NdeI-BamHI* and the fragment containing the *rdg(D53E)* coding sequence was ligated into the same sites on pET11c to generate pETR*. pET11c and pETR* were used to transform Rosetta pLysis cells (Novagen). Cultures (50 ml) were inoculated from 4 ml overnight cultures, grown to OD_{600} = 0.4, and induced with 1 mM IPTG for 4 h at 30°C. Cells were harvested at 3,300 x g and resuspended in TA buffer containing 1 tablet of Complete™ protease inhibitor cocktail (Roche). Cells were lysed in tubes containing 0.5 mm zirconia/silica beads (BioSpec Products) by agitation 5 x for 15 sec with a frequency of 30 (1/s) in a bead beater. Cell debris was pelleted twice at 13,000 x g for 30 min at 4°C and supernatant was passed through a 0.45 μm nitrocellulose filter. Protein concentrations were quantified using Bio-Rad Protein Assay Kit.

Extracellular complementation

S. coelicolor bld strains and RdgR(D53E) overexpressing strains were streaked out in close proximity on R2YE plates at 30°C. Growth of aerial hyphae induced by extracellular complementation was observed every 12 h (Willey *et al.,* 1991).

Ethylacetate extraction of *Streptomyces* solid cultures

For ethylacetate extractions, fresh spores of *ramR* mutant and *ramR* overexpressing *rdgR(D53E)* (pSNP5) strains were pregerminated (Kieser *et al*., 2000) and inoculated onto R2YE medium. After 5

days of incubation at 30°C, mycelium and spent agar medium were homogenized through a 50 ml syringe. The homogenate was subsequently extracted with one volume of ethylacetate at pH 4.5 for 4 h at RT under constant shaking. Extracts were, then, centrifuged at 5,000 x g for 15 min at RT and the ethylacetate phase was lyophilized by speed vacuum. Pellet was resuspended in 1 ml of methanol and centrifuged twice at 10,000 x g for 30 min at RT to eliminate insoluble material. For the AHF induction assay, paper disks, soaked with 20 μl of extract in methanol, were put on the surface of a 3 days old convergent mycelium of the *ramC* mutant, used as reporter strain. After methanol was allowed to evaporate under laminar hood, the reporter strain was reincubated at 30°C and monitor for AHF every 12 h.

Electron microscopy

Low temperature scanning electron microscopy was carried out as previously described (Müller *et al.,* 1991).

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Chapter II

Genome-wide analyses of *S. coelicolor* revealed that induction of aerial growth by SapB activated a developmental program needed for aerial hyphae morphogenesis

[Ω]Salvatore San Paolo; Jianqiang Huang*; Joanne M. Willey**; Stanley N. Cohen* and Charles J. Thompson[§]

Department of Molecular Microbiology, Biozentrum, Universität Basel, Basel Switzerland.

Ω Author order not yet finalized

* Department of Genetics, Stanford Medical School, Stanford, CA, USA

** Department of Biology, Hofstra University, NY, USA

§ Department of Microbiology and Immunology, Life Sciences Center, University of British Columbia, Vancouver, B. C., Canada

Running title: SapB activates the morphogenetic program for aerial hyphae formation in S. coelicolor

Key words: aerial hyphae, differentiation, SapB, biosurfactant, hydrophobins, synexpression group.

Summery

The filamentous bacterium *Streptomyces coelicolor* exhibits a complex life cycle in which initiation of aerial growth (AHF) coincides with the synthesis of SapB, a morphogenetic compound. SapB is a lantibiotic-like peptide that is derived by posttranslational modifications of the *ramS* gene product. Application of purified SapB to the developmentally delayed *ramS* mutant restored its ability to erect aerial hyphae and sporulate, strongly suggesting that it could trigger transduction events needed for morphogenesis. Using microarray and quantitative real time RT-PCR analyses we found that initiation of aerial growth, induced by SapB, provoked a rapid increase in the transcriptional abundance of morphogenetic genes, such as *rdlAB* and *chpABDFG*. A slower response and lower levels of induction were also observed for *chpH*, *chpC*. The expression of *chpE* was weakly stimulated by SapB. In addition to *rdlAB* and *chpABCDFGH*, transcript abundance of previously uncharacterized genes, encoding either hydrophobic proteins (*sco0323*, *sco1076*, *sco2703*, *sco4002*, *sco4902*), enzymes involved in cell wall biosynthesis (*sco2983*), or in production of terpene compounds (*sco7700* and *sco7701*) was also significantly upregulated between 2 h and 4 h of aerial growth. Conversely, transcriptional levels of genes possibly involved in the regulation of the energy metabolism (*narG2H2*, *sco0179*) or encoding putative transcription factors (*sco0166*, *sco0168*) were rapidly downregulated. These results demonstrated that SapB, through its biosurfactant activity can trigger the activation of a morpgohenetic program to the emerging aerial hyphae. Future investigations will clarify the role(s) played by the yet uncharacterized SapB-modulated genes during development of *S. coelicolor*.

Introduction

Although filamentous fungi and filamentous bacteria (i.e. actinomycetes, which include *Streptomyces* spp.) have diverged early in evolution, they have adopted analogous strategies for differentiation and dispersion in the environment. Both groups initially form a branched substrate or vegetative mycelium consisting of filaments which grow at their tips. In response to several physiological and stress signals, both filamentous fungi and filamentous bacteria develop specialized hydrophobic aerial hyphae that leave the hydrophilic environment of the embedded vegetative mycelium to grow upright into the air. Formation of aerial hyphae was described as a two-step process (Wösten *et al.,* 1999; Wösten and Willey, 2000). In a first step, hyphae lower the aqueous tension at the air-water interface to escape the aqueous environment of the substrate mycelium. In a second step, emerging aerial hyphae become coated with one or more hydrophobic layers, structurally similar to a mosaic of parallel rods (rodlet), which provide them with rigidity, protection and hydrophobicity. This change in surface hydrophobicity may prevent a collapse of aerial hyphae into the moist substrate as well as facilitate attachment to hydrophobic surfaces during the invasive growth of organic debris (Talbot *et al.,* 1993; Claessen *et al.,* 2002)

Filamentous fungi differentiate aerial structures through the use of surface-active low molecular weight proteins called hydrophobins (Wösten 2001; Wösten and de Vocht, 2000). Hydrophobins are small amphiphilic secreted proteins (about 100 amino acids) that possess similar hydropathy profiles and solubility characteristics although they do not share any overall sequence conservation. Nevertheless they seem to be functionally related as they can substitute for each other (Kershaw *et al.,* 1998; van Wetter *et al.,* 2000). Hydrophobins trigger aerial growth by self-assembling at the air-water interface, thus reducing dramatically the water tension on the surface of the vegetative mycelium.

Aerial hyphae formation (AHF) has been extensively studied also in the filamentous bacterium, *Streptomyces coelicolor*. Genetic analyses have revealed that such developmental switch requires the activation of a signalling cascade mediated by the *bld* genes (Chater 1993; 2001). Mutants in these genes are defective in AHF on complex medium (R2YE), in part, because they are unable to synthesize the morphogenetic compound SapB. This is an amphiphilic lantibiotic-like molecule (Kodani *et al.,* 2004) that, similar to hydrophobins, lowers the water tension of the aqueous environment from 72 to 32 mJ/m^2, enabling aerial hyphae to breach the air-water interface (Tillotson *et al.,* 1998). SapB is derived from the precursor prepeptide RamS that undergoes extensive posttranslational modifications including synthesis

of intramolecular thioether lanthionine bridges (Kodani *et al.,* 2004). *ramS* is a member of the *ramCSAB* operon, whose transcription is developmentally regulated by the response regulator, RamR (Kodani *et al.,* 2004; Nguyen *et al.,* 2002). While both the *bld* mutants and the *ramS* and *ramR* mutants are defective in the biosynthesis of SapB, these *ram* mutants undergo delayed aerial hyphae formation and sporulation on R2YE medium (Nguyen *et al.,* 2002). Furthermore, SapB is dispensable for aerial hyphae formation on minimal media (Willey *et al.,* 1991), strongly suggesting that morphological differentiation can be supported by alternative pathways in *S. coelicolor* (for a review see Talbot, 2003; see chapter I).

Aerial hyphae and spores of *S. coelicolor* have several hydrophobic surface layers, one of which is the rodlet (Wildermuth *at al.,* 1971; Wösten and Willey, 2000; Claessen *et al.,* 2002). This is made up of a mosaic of 8- to 10-nm wide rods and its formation depends on the expression of two amphipathic homologous secreted proteins, rodlins RdlA and RdlB (Claessen *et al.,* 2002). *rdl* genes are conserved in *Streptomyces* spp. and are expressed in the emerging aerial hyphae (Claessen *et al.,* 2002; 2004). However, although disruption of both *rdlA* and *rdlB* in *S.coelicolor* abolished the formation of the rodlet layer and reduced the ability to adhere on hydrophobic surfaces, the *ΔrdlAB* mutant did not show any defect in aerial hyphae formation, differentiation and overall hydrophobicity.

This led to further investigations and to the identification of the *Streptomyces coelicolor* hydrophobic aerial proteins or chaplins (Claessen *et al.,* 2003; 2004; Elliot *et al.,* 2003). Chaplins consist of a group of eight secreted hydrophobic proteins, ChpA-H, whose expression is developmentally regulated in wild type *S.coelicolor* and completely abolished in *bld* mutants. Chaplins are associated with the surface of aerial hyphae and they all share a region of similarity, the chaplin domain, composed of 60-65% of hydrophobic residues. Three long chaplins, ChpA-C (210-230 amino acids) possess two chaplin domains. The second group of short chaplins, ChpD-H (50-63 amino acids) contain only one single chaplin domain. Self-assembly of these amphiphilic proteins into an amyloid-like hydrophobic film, which causes a drop in the water tension from 72 to 26 mJ/m^2 facilitates erection of aerial hyphae. The involvement of chaplins in morphogenesis on both rich and minimal medium was confirmed by gene deletion. Deletion of all eight *chp* genes abolished aerial growth; deleting subsets of *chp* genes resulted in delays in aerial growth (Claessen *et al.,* 2003; 2004; Elliot *et al.,* 2003).

In addition to SapB and ChpA-H, other morphogenetic compounds, such as SapT from *Streptomyces tendae* (Kodani *et al.,* 2005) and the hydrophobin protein SC3 from *Schizophillum*

commune (Tillotson *et al.,* 1998, Kodani *et al.,* 2005) were able to induce AHF in several blocked developmental mutants of *S. coelicolor*.

In the present study, microarray and quantitative real time RT-PCR analyses of a SapB induced *ramS null* mutant show that SapB integrates its biosurfactant function (Tillotson *et al.,* 1998) with the activation of signaling events that up- and downregulate expression of a specific set of genes, coincident with initiation of aerial growth. We identified *chpABCDFGH* and *rdlAB* as members of SapB regulon confirming that initiation of aerial growth in response to SapB is accompanied by the activation of the morphogenetic program needed for aerial hyphae differentiation.

Results

Genome wide analyses of the expression profiles in the *ramS* mutant strain during initiation of aerial growth induced by purified SapB

Recent studies (Kodani *et al.,* 2005; our unpublished data) demonstrated that application of purified SapB to the *S. coelicolor ramS null* mutant (Nguyen *et al.,* 2002) restored its ability to complete aerial hyphae formation and sporulation. This strongly suggested that, while emerging into the air upon SapB induction, aerial cells could detect changes in their local environment, which triggered signaling events leading to morphogenesis. To test this hypothesis, we performed microarray analyses, comparing the expression profiles of the *ramS* mutant induced with purified SapB to the uninduced strain.

Synchronously pregerminated spores from the *ramS* mutant were inoculated on cellophane discs on the surface of R2YE agar plates and incubated at 30°C. After 36 h of growth, purified SapB (at a concentration of 1.5 μM) was added and initiation of aerial growth was monitored over time by scanning electron microscopy (Fig. 1). Tips of a few sparse emerging aerial hyphae were visible even after 1 h of induction, suggesting that SapB rapidly prompted events required for AHF. Aerial growth progressively continued over time until the vegetative mycelium of the SapB-induced *ramS* mutant was fully covered with loosely coiled but still unseptated aerial hyphae, 8 h after induction (Fig. 1). Sporulation was detected only after prolonged incubation at 30°C (data not shown). Unlike the SapB-induced *ramS* mutant, uninduced *ramS* developed aerial filaments with a delay of 24 h (Fig. 1; data not shown).

To compare the expression profiles of genes differentially expressed in the SapB-induced *ramS* mutant relative to the uninduced strain, RNA samples were extracted at different time points (0.5-8 h; Fig. 1) and analyzed by microarray experiments. Significant differences (higher then two-fold) in the levels of gene expression were observed after 3 h of induction (Fig. 2; data not shown). Most of the SapB-upregulated genes encoded putative secreted hydrophobic proteins. These included the mophogenetic genes *chpAEC*, *rdlAB*, and the previously uncharacterized genes *sco0323, sco1076*, *sco2703, sco4902* and *sco4002* (Fig. 2; Fig. 3), suggesting that SapB activated signaling events able to increase the overall hydrophobicity on the surface of the vegetative mycelium (e.g. *chpAEC*) and was required to form the rodlet layer on the emerging aerial filaments (*rdlAB*). In addition to this group of genes, increased transcriptional levels were observed in genes encoding putative products likely involved in the cell wall biosynthesis (*sco2983*) and in the synthesis of terpene compounds (*sco7700* and *sco7701*) (Fig. 2; Fig. 3).

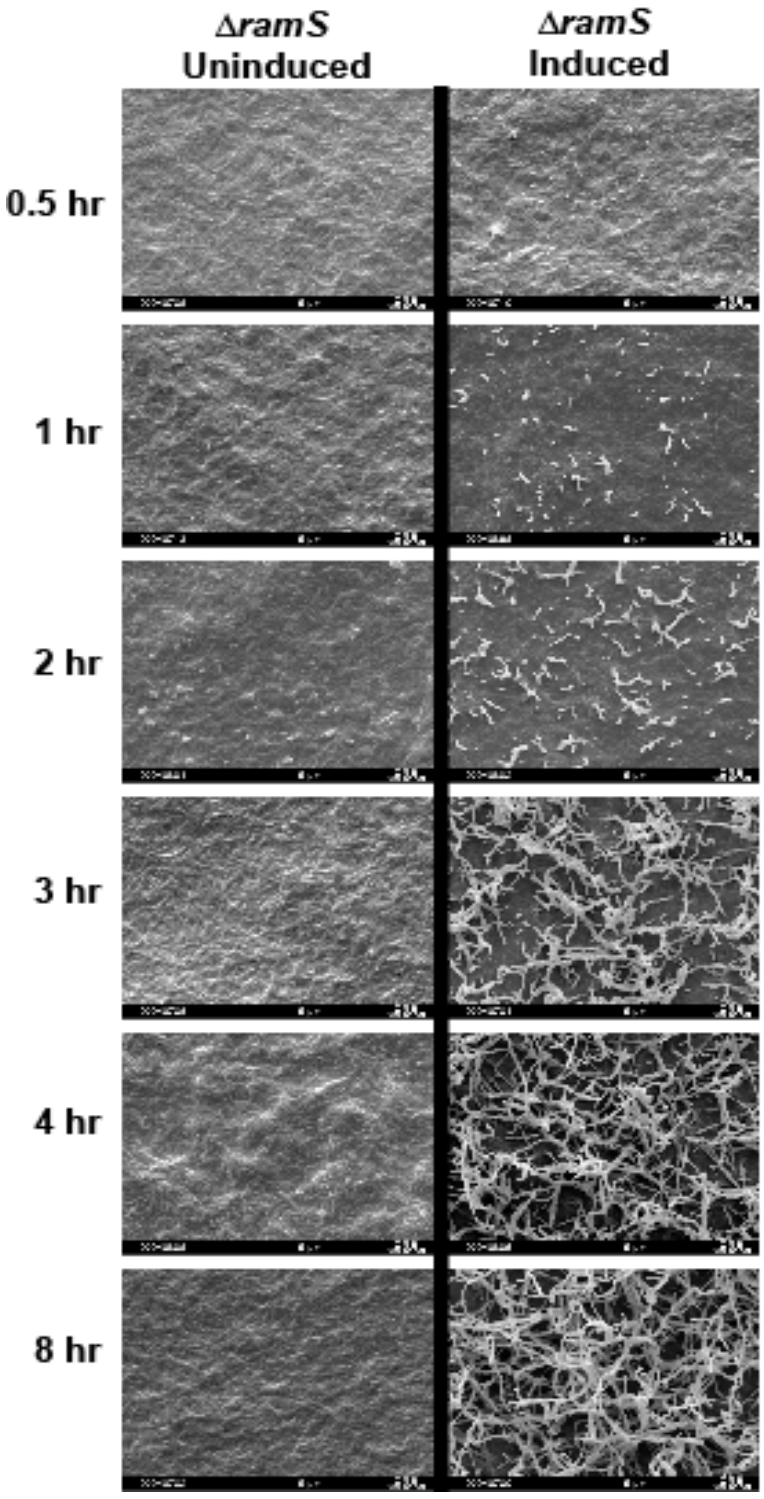

Fig. 1. Induction of aerial hyphae formation in the *ramS null* mutant with purified SapB. Pregerminated spores from the *ramS* mutant were inoculated on cellophane discs on R2YE medium and incubated at 30°C for 36 h. Scanning electron micrographs showed that the application of 1.5 μM of purified SapB induced the erection of a few sparse aerial hyphae beginning after 1 h of induction, until the vegetative mycelium lawn was fully covered with coiled but unseptated aerial hyphae after 8 h, (right column). Unlike SapB, application of the incubation buffer, used as negative control, did not induce any morphological switch (left column).

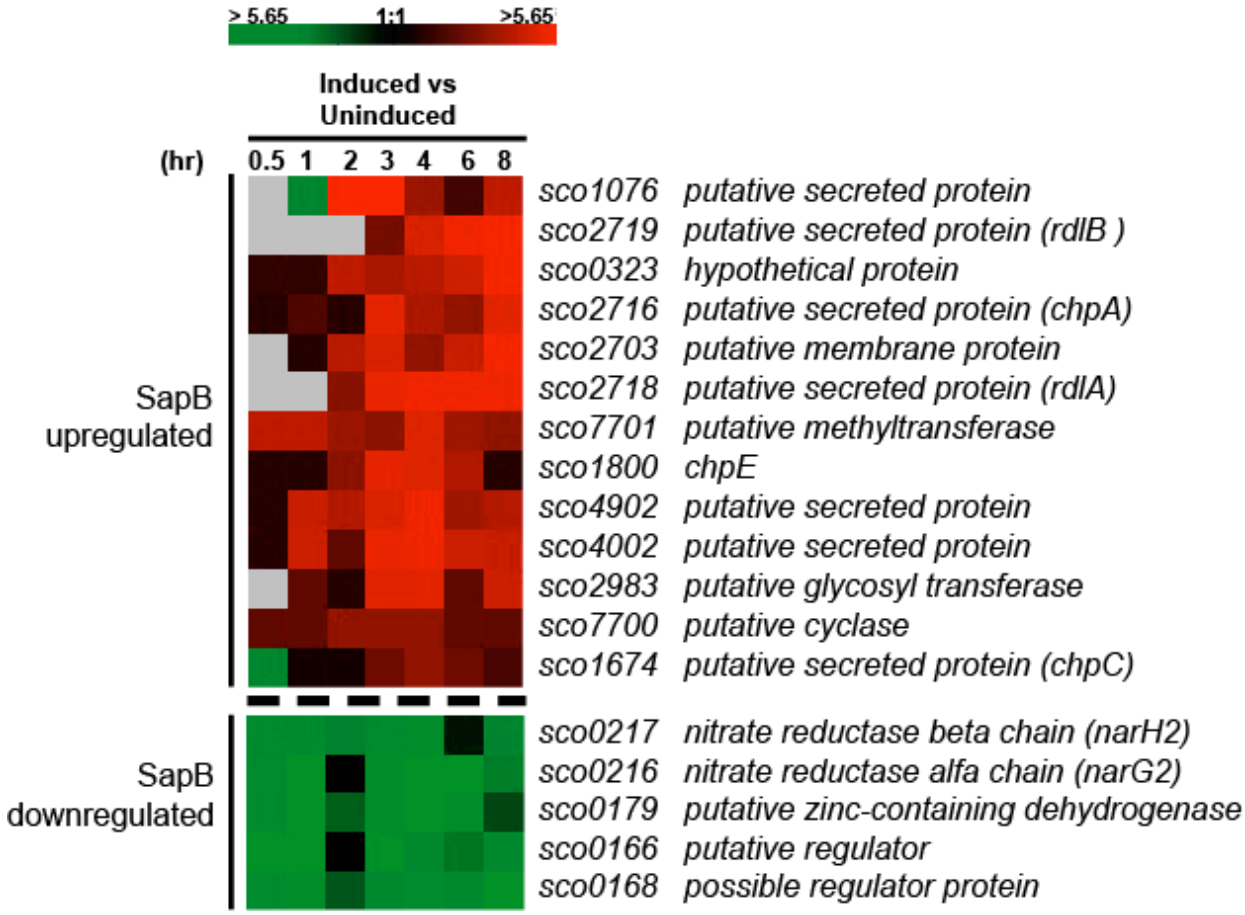

Fig. 2. Genome wide analyses identify SapB-modulated genes.
Profiles indicate the levels of gene expression in the *ramS* mutant induced with purified SapB relative to the uninduced control strain. RNA was extracted at the indicated time points from the solid R2YE cultures as described in Fig. 1. For microarray experiments, Cy5-dCTP (red)-labelled cDNA samples from the SapB-treated *ramS* mutant were hybridized with the same time point Cy3-dCTP (green)-labelled cDNA samples of uninduced *ramS*. Genes were clustered hierarchically according to similarity in the expression profiles (Huang *et al.,* 2001); rows correspond to individual genes and columns correspond to successive time points. The change in transcript abundance for each gene is displayed on a colour scale, in which colour saturation represents the magnitude of the difference of RNA abundance. The brighter red shades represent higher transcript abundance and brighter green shades represent lower transcript abundance relative to the uninduced control *ramS* mutant strain. Black indicates an equal amount of RNA abundance in induced vs uninduced, and grey represents absence of data. Ratios of genes with multiple spots on the array were averaged.

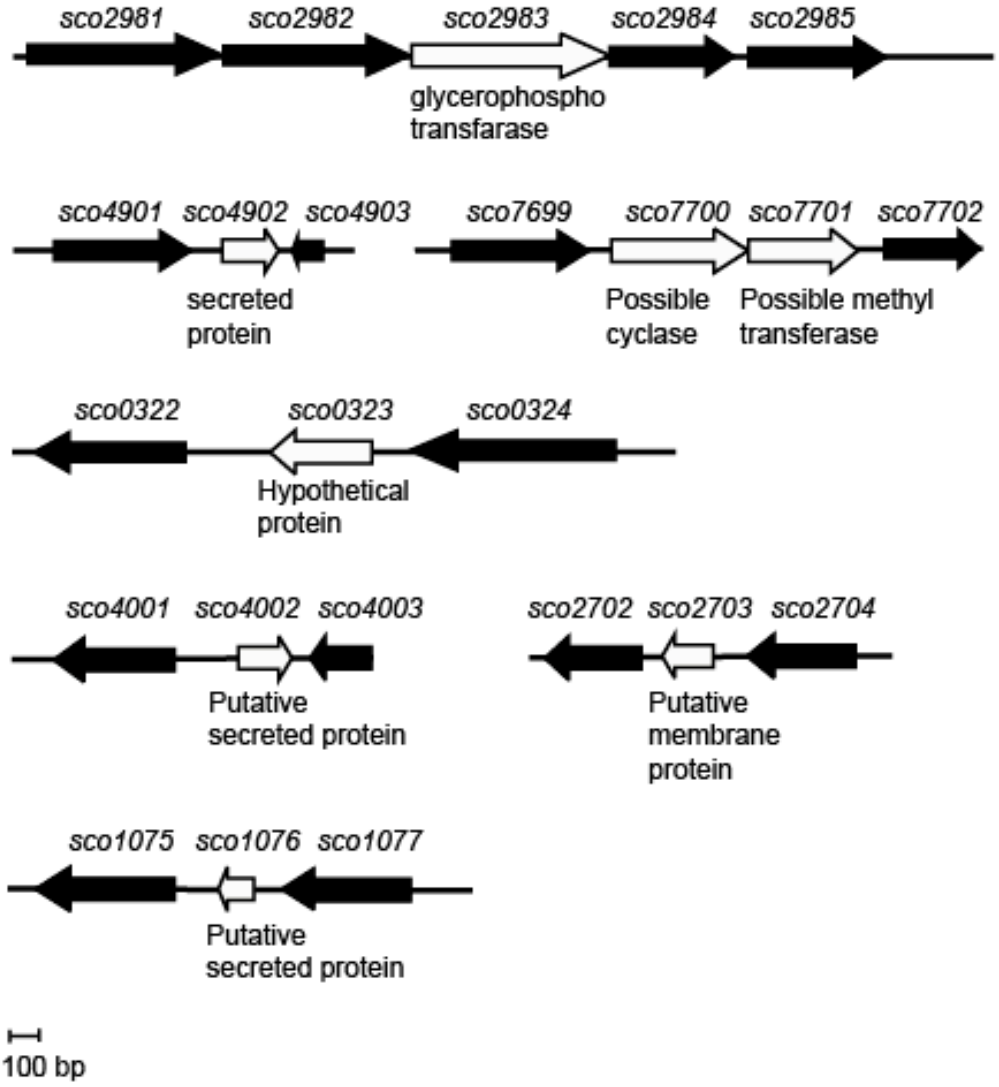

Fig. 3. Genetic organization of yet uncharacterized SapB-upregulated genes potentially involved in aerial hyphae differentiation in *Streptomyces coelicolor*.
Arrows indicate the orientation of each gene within each locus. The white arrows correspond to the SapB-upregulated target genes, while the black arrows represent the flanking open reading frames.

In agreement with the results of these microarray analyses, upregulation in the expression profiles of most SapB-induced genes (*sco0323*, *sco2703*, *sco7700*, *sco7701*, *rdlAB*, *chpACE*) was also found in microarray experiments of the wild type strain of *S. coelicolor*, J1501, at the onset of AHF, coincident with SapB biosynthesis, during development under the same conditions (data not shown).

Developmental processes based on the formation of new specialized cells may involve repression of specific genes. Initiation of aerial growth induced by SapB downregulated the transcriptional levels of another group of target genes, including *narG2H2, sco0179, sco0166* and *sco0168* (Fig. 2). The putative products of these genes may be involved in the regulation of the energy metabolism (*narG2H2* and *sco0179*) (van Keulen *et al.,* 2005) or may be transcriptional regulators (*sco0166* and *sco0168*) (Fig. 2; Fig. 7B). Coordinately with the SapB-induced genes, downregulation of the SapB-repressed genes, *narG2H2* and *sco0179*, was also observed during the onset of aerial hyphae formation and SapB biosynthesis in microarray analyses of J1501 (data not shown).

SapB coordinates expression of *chpABCDEFGH* and *rdlAB* throughout aerial hyphae morphogenesis.

Microarray analyses showed that initiation of SapB induced aerial growth triggered expression of *chpACE* (Fig. 2). Chaplins consist of a group of eight homologous genes whose expression is developmentally regulated and coincident with aerial hyphae formation in wild type *S. coelicolor* (Claessen *et al.,* 2003; Claessen *et al.,* 2004; Elliot *et al.,* 2004). To confirm the results of the microarray analyses and test whether SapB could promote the expression of *chp* genes, the relative transcriptional abundance of *chpABCDEFGH* was measured by quantitative real time RT-PCR (qRT-PCR) analyses, using RNA samples used for the microarray experiments.

qRT-PCR experiments confirmed that, in addition to *chpAC*, SapB upregulated the transcriptional levels of *chpBDFGH* (Fig. 4A, B; Fig. 6). In particular, transcription of different *chp* genes exhibited different kinetic profiles and maximal levels of transcript abundance in response to SapB. Based on these data, we grouped *chp* genes into two SapB induction groups (Fig. 4A, B; Fig. 6). *chp* of group A included the fast SapB responding genes, *chpABDFG* (Fig. 4A; Fig. 6). Kinetic profiles of SapB induction group A were characterized by an increase higher than 2-fold between 2 h and 4 h after the initiation of aerial hyphae formation, with maximal increases of 3 to 8-fold after 4 h of AHF. *chp* genes of SapB induction group B (*chpCH*) showed a

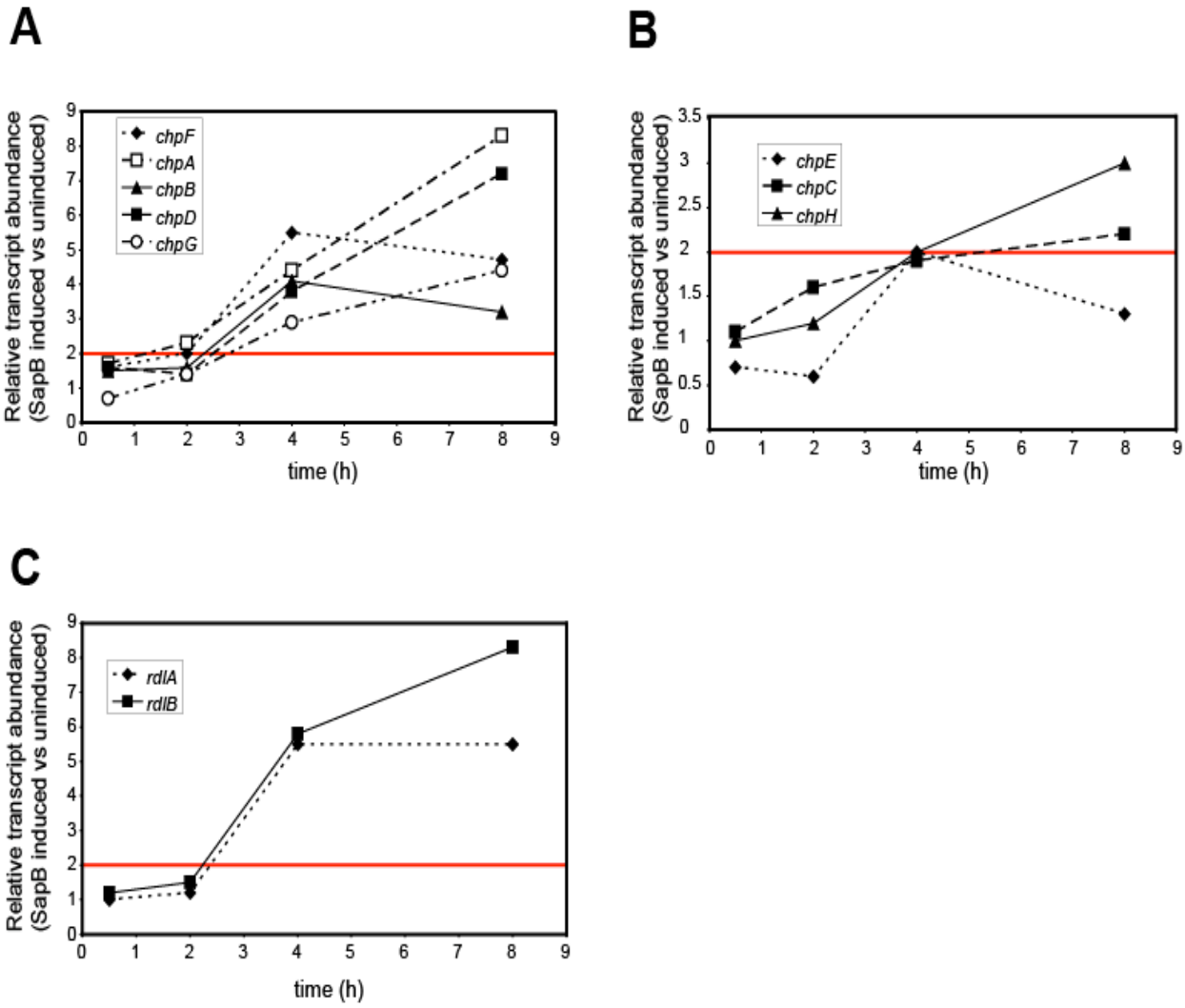

Fig. 4. Kinetics of relative transcript abundance of *chpABCDEFGH* and *rdlAB* upon induction with purified SapB.
RNA samples used for these experiments were the same as those used for the microarray analyses (Fig. 2). The amount of each cDNA tested was internally normalized to the 16S rDNA and relative transcript abundance for each gene was reported as a ratio of normalized values. *chpABDFG* (A) and *rdlAB* (C) showed a rapid response to SapB, reaching transcriptional increases higher then 2-fold between 2-4 h of aerial hyphae formation (induction group A). *chpCH* (induction group B) exhibited a slower response to SapB and transcriptional increases higher than 2-fold after 4 h (B). Only a transient maximal transcriptional level of 2-fold was shown by *chpE* (B). Red line indicates the significance of the response to SapB as established by the 2-fold upregulation limit.

slower response to SapB and transcript abundance kinetics that reached a 2 to 3 fold increase only between 4 h and 8 h of aerial growth (Fig. 4B; Fig. 6). The only *chp* gene that exhibited a more transient response to SapB, was *chpE*, whose level of transcript abundance was maximal (about 2-fold) at 4 h after the onset of AHF (Fig. 4B; Fig. 2). Moreover, since its kinetic profile did not match with any of the other *chp* genes, *chpE* could not be included in either of the two SapB induction groups. This result was in agreement with the observation that *chpE* is constitutively expressed throughout development in the wild type strain of *S. coelicolor* with a slight increase exhibited at the onset of AHF (Claessen *et al.,* 2003; data not shown).

Similar to the fast SapB responding *chp* genes (SapB induction group A *chp*) *rdlA* and *rdlB* transcription displayed a rapid increase of 2-6 fold between 2-4 h after the initiation of aerial hyphae formation (Fig. 4C; Fig. 6) and maximal levels after 4 h of aerial growth. Such similarity in their temporal expression profiles strongly suggested that both *chpABDFG* and *rdlAB* belonged to the same SapB induction group and that a common regulatory pathway may coordinate their expression during aerial hyphae morphogenesis.

SapB-induced aerial hyphae formation activates at least three regulatory pathways.

In addition to *chpACE* and *rdlAB*, microarray analyses showed that another group of genes, *sco0323*, *sco1076*, *sco2703*, *sco2983*, *sco4002*, *sco4902*, *sco7700* and *sco7701*, was upregulated upon the onset of aerial growth in response to SapB (Fig. 2). Once again, qRT-PCR experiments not only confirmed the results of microarray analyses, but also showed that the temporal expression profiles for these genes were very similar to those of either *chpABDFG-rdlAB* or *chpCH* (Fig. 4; Fig. 5). In particular, like *chpABDFG* and *rdlAB* (SapB induction group A), the SapB-upregulated genes, *sco2983*, *sco4902*, *sco7700* and *sco7701* exhibited a fast transcriptional response to SapB with relative transcript abundances that reached increases higher than 2-fold between 2-4 h and maximal levels of 3 to 8-fold after 4 h of aerial hyphae formation (compare fig. 4A and 5A; Fig. 6). Genes, *sco0323*, *sco1076*, *sco2703* and *sco4002* showed a slower response to SapB. Analogously to the SapB induction group B (*chpCGH*), they exhibited transcriptional profiles that reached levels higher than 2-fold only between 4 h and 8 h of aerial growth (compare Fig. 5B and 4B; Fig. 6).

Initiation of aerial hyphae formation upon SapB induction decreased the transcriptional abundances of *sco0166*, *sco0168*, *sco0179*, *narG2H2*, as shown by microarray analyses (Fig. 2) and confirmed by

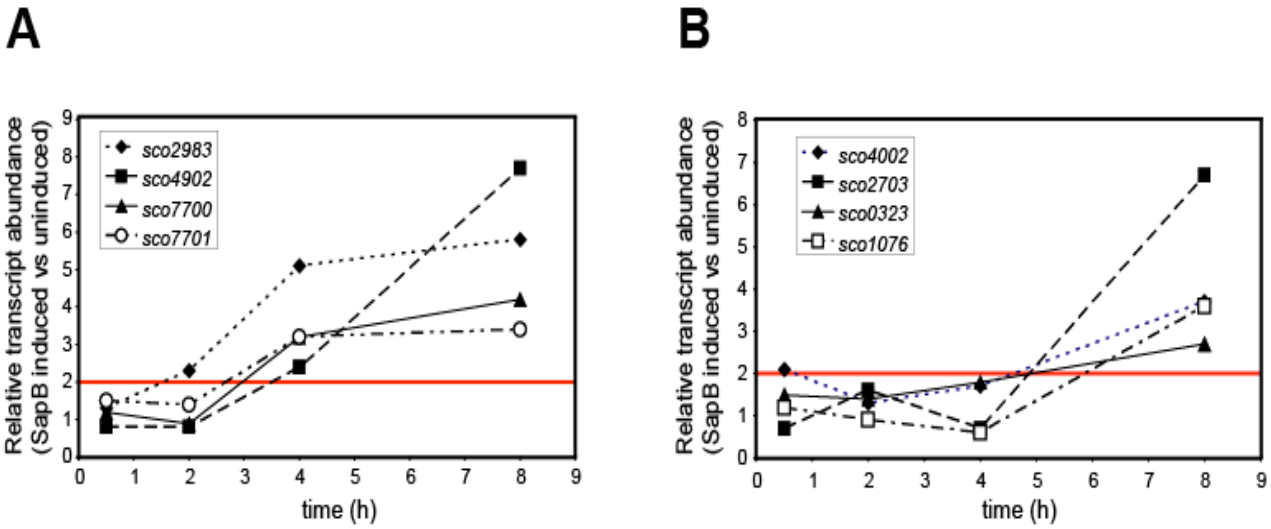

Fig. 5. Kinetics of relative transcript abundance identify two induction groups for the SapB-upregulated genes.
RNA samples used for these experiments were the same as were used for the microarray analyses as (Fig. 2). The amount of each cDNA tested was internally normalized to the 16S rDNA and relative transcript abundance for each gene was reported as a ratio of normalized values. The red line indicates the significance of the response to SapB as established by the 2-fold upregulation limit. (A) Temporal expression profiles of *sco2983*, *sco4902*, *sco7700* and *sco7701*. Similar to *chpABDFG* and *rdlAB*, these genes showed a rapid response to SapB and expression levels increased more than 2-fold between 2-4 h after initiation of AHF (synexpression group A) (compare with Fig. 4A, C).
(B) Temporal expression profiles of *sco0323*, *sco1076*, *sco2703* and *sco4002*. Similar to *chpCH*, these genes exhibited a slower response to SapB and maximal transcriptional abundances higher than 2-fold only after 4 h of induction (synexpression group B) (compare with Fig. 4B).

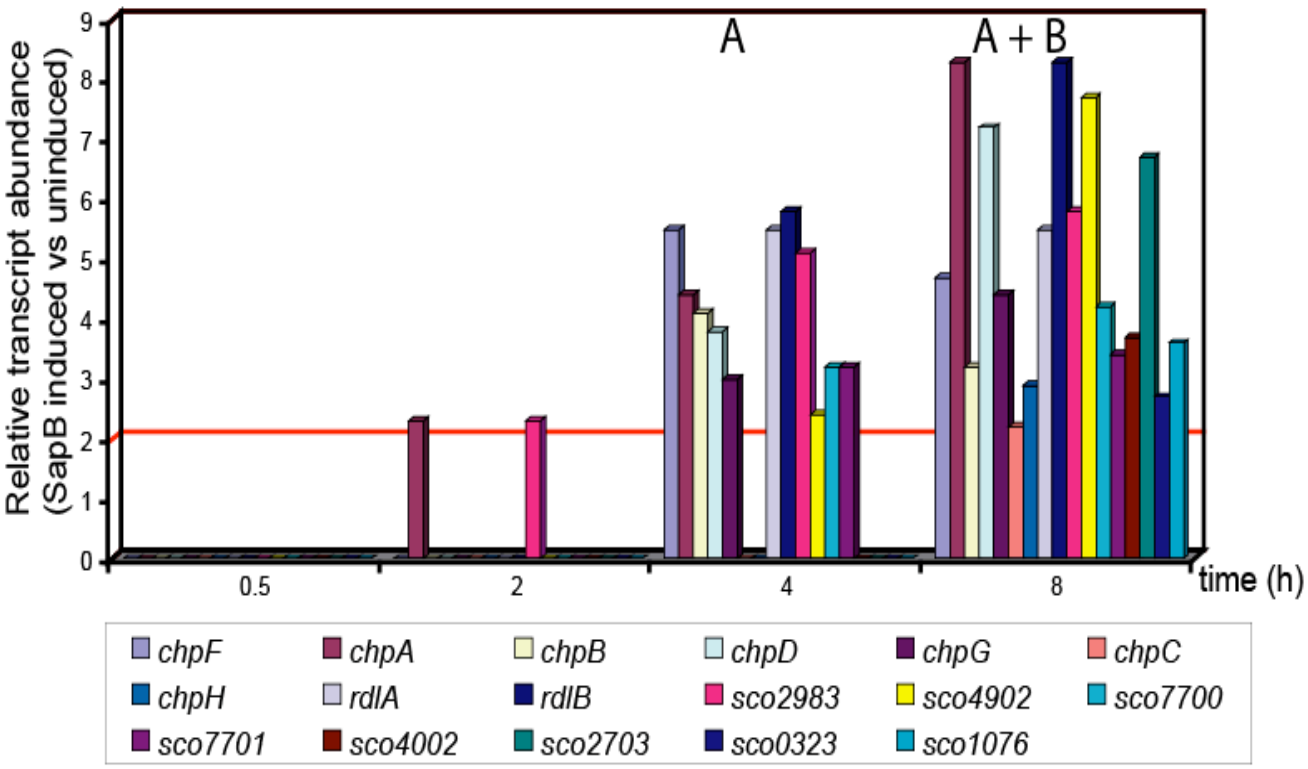

Fig. 6. Histograms representing the SapB induction groups A and B.
Genes of the two SapB induction groups, having transcriptional increases higher than 2-fold (red line) were represented in histograms. All genes of group A display transcriptional increases higher than 2-fold between 2 h and 4 h after initiation of aerial hyphae formation. Genes of group B showed transcriptional increases higher than 2-fold only at 8 h. A indicates genes of the SapB induction group A; A + B corresponds to both genes of group A and group B.

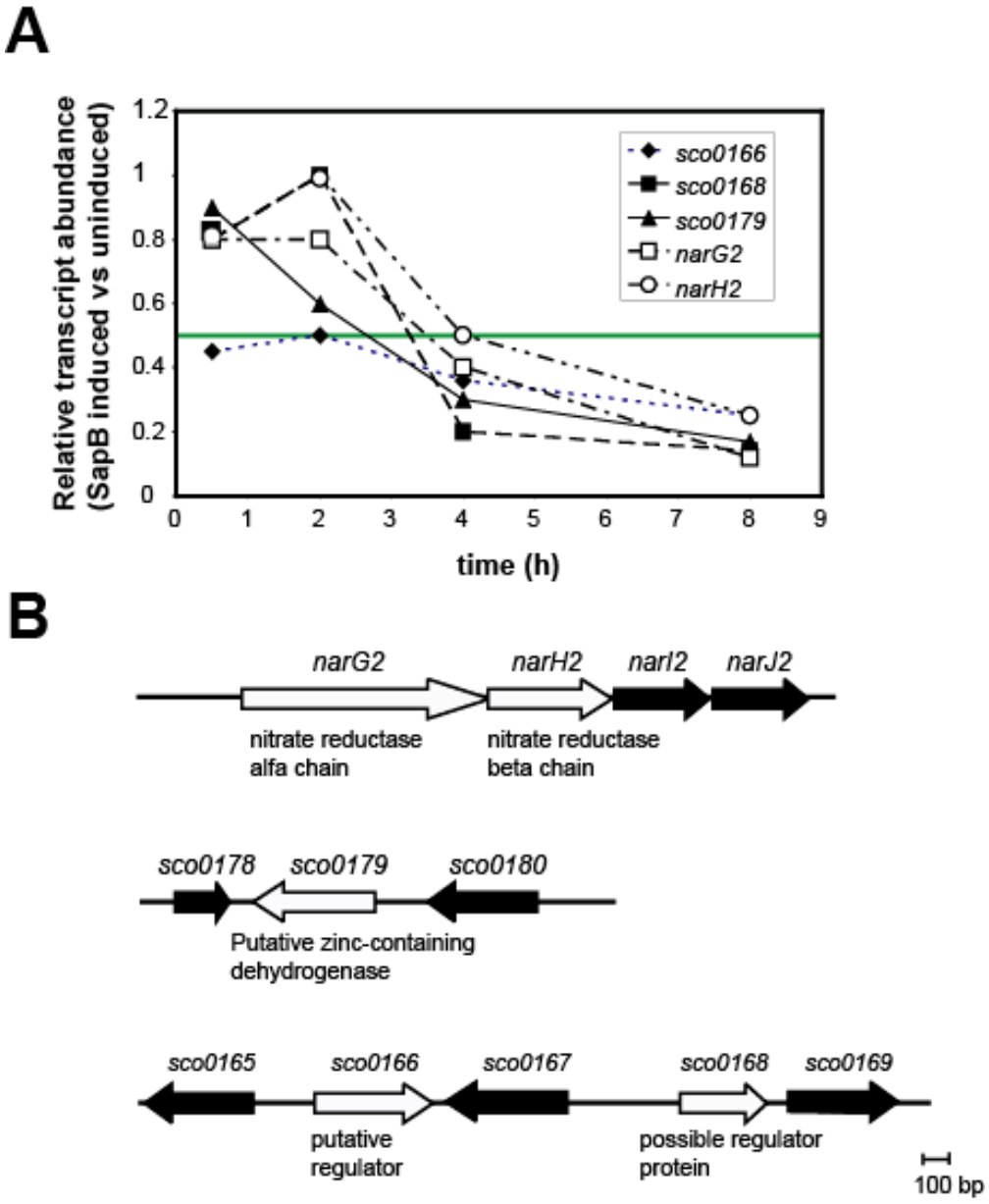

Fig. 7. Expression profiles and genetic organization of the SapB-downregulated genes.
RNA samples used for these experiments were the same as were used for the microarray analyses (Fig. 2). The amount of each cDNA tested was internally normalized to the 16S rDNA and relative transcript abundance for each gene was reported as a ratio of normalized values.
(A) Kinetics of relative transcript abundance showed that all the SapB-downregulated genes (except sco0166; see text) exhibit a rapid drop of 2-5 fold in transcription between 2 h and 4 h after AHF. Green line indicates the significance of the response to SapB as established by the 2-fold downregulation limit.
(B) Genetic organization: arrows indicate the orientation of each gene in each locus. The white arrows correspond to the SapB-downregulated target genes, while the black arrows represent the flanking open reading frames.

PCR experiments (Fig. 7A). Coordinately with the genes of the SapB induction group A, this group of SapB-downregulated genes (with the exception of *sco0166*) showed a prompt response to SapB with a rapid dropof about 2-5 fold in their transcriptional levels between 2 h and 4 h from the initiation of aerial hyphae formation. A different expression profile, however, was exhibited by *sco0166,* whose transcriptional level significantly decreased immediately after the addition of SapB when erection of aerial hyphae was not yet visible by scanning electron microscopy (time point 0.5 h in Fig. 1; Fig. 7A), suggesting that downregulation of this gene might be achieved through the activation of a different regulatory pathway.

Together these results suggested that initiation of aerial hyphae formation upon SapB induction coincided with the activation of at least three regulatory circuits: two leading to the transcriptional increase of the SapB-upregulated genes and one leading to the transcriptional decrease of the SapB-downregulated genes). Roles in development for some of the SapB modulated genes, such as *chpABCDEFGH* and *rdlAB*, had been described (Claessen *et al.,* 2002; 2003; 2004; Elliot *et al.,* 2003). Possible roles that might be played by the still uncharacterized SapB-modulated genes will be discussed.

The predicted products of *sco4902* and *sco1076* show structural similarities to Rodlins and Chaplins.

Initiation of aerial growth in the *ramS null* mutant upon induction with puified SapB activated the expression of a group of genes (*sco4002*, *sco4902* and *sco1076*) encoding, like *chpABCGEFGH* and *rdlAB*, putative secreted proteins (Fig. 2; Fig. 5). The predicted products of these genes do not share any amino acid sequence conservation with each other, or with chaplins or rodlins. However, similar to chaplins and rodlins, they are rich in hydrophobic amino acids (about 54-63%) and have similar N-terminal signal peptide sequences, as determined with SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0) (not shown). Furthermore, structural similarities were predicted for sco*1076* and *sco4902* by the analyses of the hydropathy patterns of their putative encoded products (Fig. 8). Like RdlA and RdlB, the 120 amino acid putative product of *sco4902* displayed three hydrophobic amino acid stretches, one at the N-terminus and two at the C-terminus (I, II, III, in Fig. 8A) separated by an amphiphilic stretch of ~20 amino acid between the hydrophobic region I and II. The 64 amino acid predicted product of *sco1076*

was similar to the short chaplins; an amphipathic amino acid stretch at its C-terminal region was almost superimposable with the chaplin domain (b in Fig. 8B).

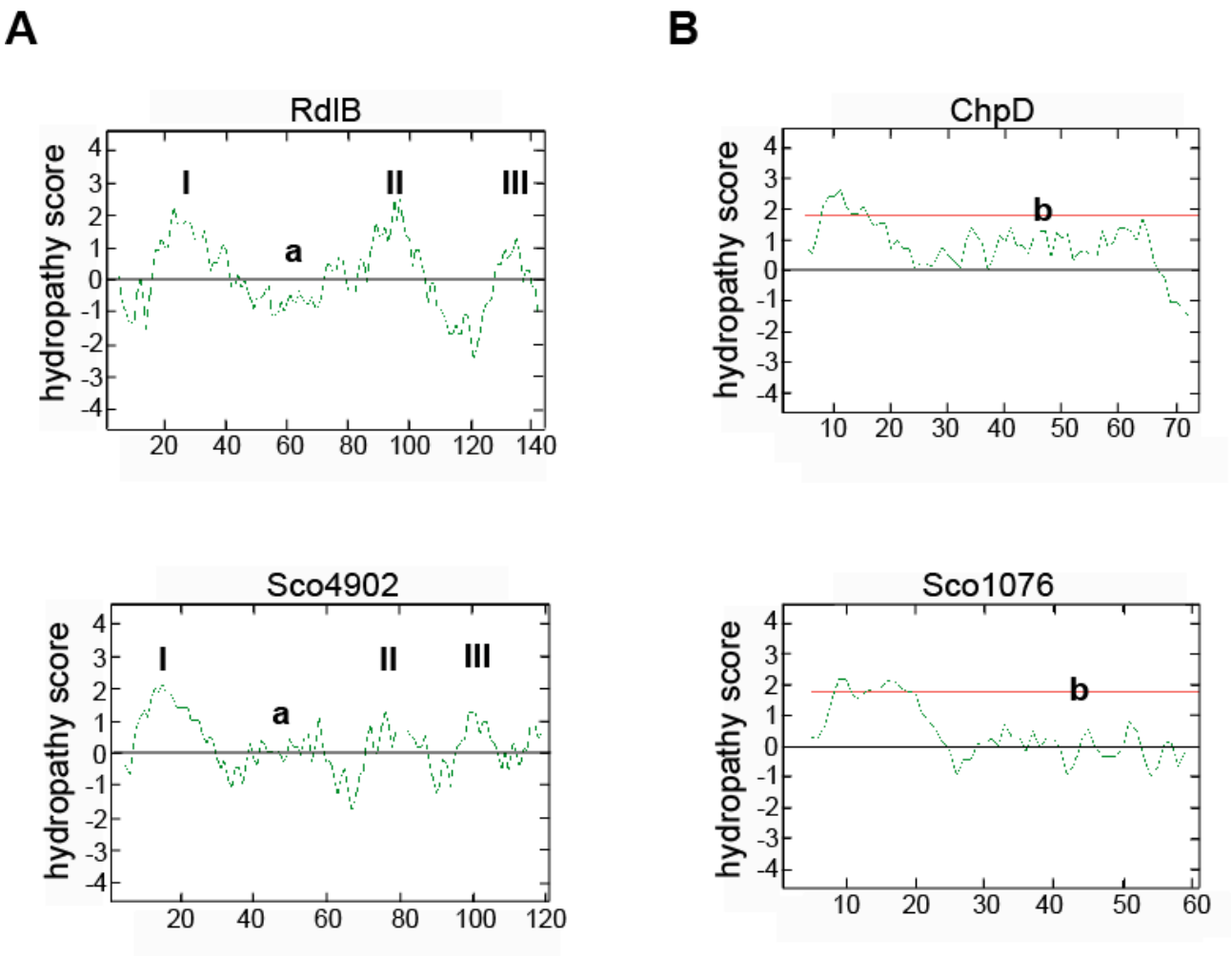

Fig. 8. Structure predictions of the putative products of *sco4902* and *sco1076*. The amino acid sequences of Sco4902 and Sco1076 predict a similar hydrophobic nature between RdlB and Sco4902 (A) or between ChpD and Sco1076 (B). I, II, III corresponds to the three hydrophobic amino acid stretches similarly arranged in RdlB and Sco4902. a indicates the amphipathic amino acid region separating the hydrophobic stretches I and II; b corresponds to the chaplin domain and to the chaplin-like domain in ChpD and Sco1076 respectively. The red line defines the hydropathy score limit for the identification of possible membrane spanning amino acid regions.

Discussion

Induction of AHF by purified SapB is accompanied by the activation of a gene expression program

To differentiate aerial structures, both filamentous fungi and filamentous bacteria have to secrete biosurfactant compounds that allow the emerging aerial hyphae to breach the air-water interface by reducing the water tension (Wösten and Willey, 2000). SapB, one of the first biosurfactant peptide isolated from *S. coelicolor* (Guijarro *et al.,* 1988) attracted interest for its role in erecting aerial hyphae (Willey *et al.,* 1991). Early studies showed that addition of purified SapB to developmentally blocked *bld* mutants restored their capacity to form aerial filaments. However, these filaments had aberrant structures and did not differentiate into spores (Tillotson *et al.,* 1998). Thus, SapB was seen as a biosurfactant that had no role as signaling molecule in differentiation. The developmental block exhibited by *bld* mutants, however, is not only due to their inability to synthesize SapB, but rather to their defects in stress and physiological response systems (Pope *et al.,* 1996; Süsstrunk *et al.,* 1998; Viollier *et al.,* 2001; Kelemen *et al.,* 2001; Viollier *et al.,* 2003). Most *bld* mutants are blocked in the perception and/or transduction of signals needed to complete morphogenesis, once hyphae are induced to grow upright by the biosurfactant activity of SapB (Kodani *et al.,* 2005). SapB is a lantibiotic-like molecule, derived from the product of the morphogenetic gene, *ramS*, a member of RamR regulon (Kodani *et al.,* 2004; Nguyen *et al.,* 2002). Unlike classical *bld* mutants, the *ramS* mutant does not have physiological defects but is severely delayed in aerial hyphae formation on complex medium (Nguyen *et al.,* 2002). It is able to complete morphogenesis when purified SapB is applied (Fig. 1; data not shown; Kodani *et al.,* 2005), strongly suggesting that, in this mutant, the biosurfactant activity of SapB may couple the initiation of aerial growth with regulatory events needed for aerial hyphae differentiation and sporulation. To assess this hypothesis, we performed microarray and quantitative real time RT-PCR experiments, comparing the expression profiles of SapB-induced *ramS* mutant relative to uninduced *ramS*. Our results showed that initiation of aerial growth led to the activation of regulatory circuits that modulated the expression of a number of downstream genes, including the morphogenetic genes *chpABCDFGH* and *rdlAB* (Fig. 2; Fig. 8). Most of the SapB-modulated genes were upregulated (*chpABCDFGH*, *rdlAB*, *sco0323*, *sco2703*, *sco7700*, *sco7701*) or downregulated (*sco0179*, *narG2H2*) in microarray analyses of the wild type strain, J1501 (not shown) at the onset of AHF, coincident with SapB biosynthesis. Together these results

provided evidence that by breaching the aqueous tension SapB exposes the hyphae on the surface of the mycelium to a radically different environment, causing changes in gene expression that enable a developmental switch needed for AHF and differentiation.

Studies of kinetics of transcript abundance showed, furthermore, that the SapB-upregulated genes could be clustered into two induction groups (group A and B) according to the rate of their transcriptional increase and their maximal responses to SapB (Fig. 4; Fig. 5). Induction groups, indeed, may correspond to genes that are under control of a common regulatory pathway, thus exhibiting similar temporal expression profiles in developmental processes (Vohradsky *et al.,* 2000; Niehrs and Pollet, 1999). It is interesting to speculate that initiation of aerial growth, triggered by SapB, activates at least two different regulatory pathways that positively modulate gene expression (Fig 9). These pathways may respond to different environmental signals perceived during the emergence of aerial hyphae into the air (a and b in Fig. 9). One pathway, triggered as soon as aerial hyphae have perceived changes at the air-water interface by means of the biosurfactant activity of SapB, would activate the expression of the genes of the induction group A, which respond rapidly to SapB (a in Fig. 9). The second pathway, which activates the expression of the slow SapB responding genes (induction group B), would be activated, instead, by downstream signals generated by the products of the group A genes and/or by signals sensed when the aerial hyphae have already left the aqueous vegetative mycelium, standing upright into the air (b in Fig. 9. Moreover, as expected from a developmental switch, initiation of aerial growth also coincided with downregulation in transcription of another set of specific target genes (pathway c in Fig. 9; Fig. 7), further supporting, that the biosurfactant activity of SapB determines changes in gene expression needed for the formation of a new specialized cell type. Additional statistical and transcriptional analyses of the SapB-modulated genes will be performed in future studies to confirm the existence and to characterize the molecular nature of the SapB-triggered transduction pathways.

Proposed roles for the members of the SapB regulon during initiation of aerial hyphae formation

Among the SapB-upregulated genes, we identified *chpABCDFGH* and *rdlAB*, previously shown to be exclusively expressed in the emerging aerial hyphae by studies of eGFP-fusions (Claessen *et al.,* 2003; 2004; Elliot *et al.,* 2003). Chaplins, in particular, increase the overall hydrophobicity on the surface of the vegetative mycelium where they form an amyloid-like hydrophobic film that lowers the air-water tension,

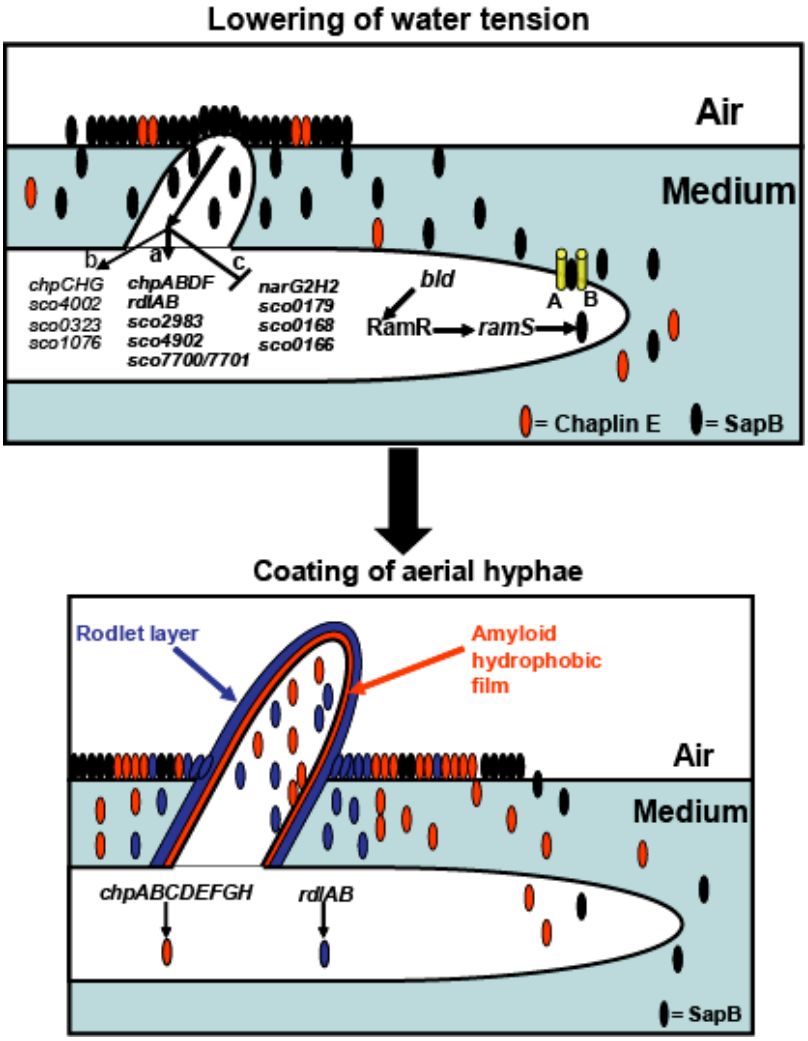

Fig. 9. SapB-induction of AHF coincides with the activation of a developmental program in *S. coelicolor*.
On complex medium, SapB represents the main pathway for erection of aerial hyphae. SapB is generated by posttranslational modifications of the product of the *ramS* gene and is exported to the surface of the embedded vegetative mycelium by the RamAB exporter (A, B in the top panel). Transcription of *ramS* is driven by the response regulator RamR, activated by the *bld* gene signalling cascade in response to environmental and physiological conditions. Self-assembling of SapB (and probably also of ChpE) lowers the water tension and allows emerging aerial hyphae to breach the air-water interface. This change in the local environment would generate signals that trigger the activation of at least three regulatory pathways (a, b, c). Pathway a (a in the top panel) leads to a rapid response to SapB and high increases in the transcriptional levels of *chpABDFG*, *rdlAB*, *sco2983*, *sco4902*, *sco7700/7701*; pathway b (b in the top panel) induces a slower response to SapB and lower transcriptional increases of *chpCH*, *sco0323*, *sco1076, sco2703* and *sco4002*; pathway c (c in the top panel) activates regulatory events that rapidly downregulate transcriptional levels of *narG2H2*, *sco0179*, *sco0168*, *sco0166*. The activation of the three regulatory pathways and thus the temporal regulation of the SapB-modulated genes may represent part of the developmental program needed for aerial hyphae morphogenesis. ChpA-H, eventually, will assemble at the cell wall-air interface into an insoluble amyloid film that provides a hydrophobic surface for the formation of the rodlet layer formed by RdlA and RdlB (low panel).

enabling aerial hyphae to escape into the air (Fig 8; Claessen *et al.,* 2003). Rodlins are required to align chaplin fibrils into rods of 8 to 10 nm to form the rodlet layer that covers the surface of aerial hyphae and spores (Claessen *et al.,* 2004). Thus, as morphogenetic compound for cell differentiation, SapB activates expression of genes that provide additional biosurfactant support for the erection of the aerial hyphae (chaplins) and that are needed for the formation of those structures (amyloid film and rodlet layer) typically associated with mature aerial filaments (Wösten and Willey, 2000; Talbot *et al.,* 2003). In addition to chaplins and rodlins, the predicted secreted products of the SapB-induced genes, *sco1076*, *sco2703*, *sco4002* and *sco4902*, might also contribute to the formation of these aerial hyphae associated hydrophobic structures as indicated by their high content in hydrophobic amino acids and by the similarity of their hydropathy profiles (*sco4902* and *sco1076* in Fig. 8) to rodlins and chaplins. Future studies will clarify whether the products of these genes, having structure similarities with chaplins and rodlins, can also serve similar functions during aerial hyphae formation.

Aerial hyphae differentiation is accompanied by a change in the cell wall structure (Wildermuth, 1970; Flärdh *et al.,* 1999). Unlike vegetative hyphae, typical aerial hyphae form unbranched and loosely coiled filaments (Fig. 1), indicative of a switch in the synthesis of the peptidoglycan at the apical parts of the hyphae and/or of modifications of existing components of the cell wall. Accompanying this change in shape and mode of growth of cells, aerial hyphae formation induced by SapB led to a rapid increase in the transcriptional levels of *sco2983*, encoding a putative glycerophosphotransferase which shows 52% identity with the teichoic acid biosynthesis protein, TagF, of *Bacillus subtilis*. Teichoic acids are a heterogeneous class of polyanions that are covalently linked to the peptidoglycan or to the glycolipids of the plasma membrane. They are essential components of the cell wall of Gram-positive bacteria and provide several functions relating to the elasticity, porosity, tensile strength of the cell wall as well as to the trafficking of metabolites, cell-cell adhesion and biofilm formation (for a review see Neuhaus and Baddiley, 2003). The observation that SapB promotes the expression of a gene (*sco2983*), encoding a TagF-like protein, suggests that changes in the cell wall structure and in the teichoic acid composition might be part of the developmental program activated by SapB, required for the erection and coiling of aerial hyphae.

Once hyphae have abandoned the embedded mycelium and have reached a radically different environment in the air, new ways of long-distance cell-cell communication are likely to be adopted to allow aerial hyphae to coordinate gene expression and physiological processes during morphogenesis.

Such aerial cell-cell signaling might be mediated by volatile organic compounds (VOCs), used by a wide variety of soil bacteria and fungi or soil bacteria and plants. These compounds can alter, in fact, gene expression as well as protein profiles in fungi and affect the growth rate and the differentiation program in both plants and fungi (Wheatley, 2002; Ryu *et al.,* 2003; Palková *et al.,* 1997). *Streptomyces* spp. produce a remarkable array of VOCs (Bentley *et al.,* 2002; Sunesson *et al.,* 1997; our unpublished data) that might play a role not only in the interaction with the surrounding environment, but also in coordinating morphological differentiation. In support of this hypothesis, extracts of VOCs from *S. coelicolor* induce AHF in the *bldK* mutant (our unpublished data). Mutants in *cyc2* (*sco6073*), required for the biosynthesis of the sesquiterpene volitile compound, geosmin, exhibit a reduced sporulation (Gust *et al.,* 2002). Further evidence was provided by our microarray and quantitative real time RT-PCR analyses. These showed that initiation of aerial growth in J1501 (data not shown) or in the *ramS* mutant upon induction with purified SapB (Fig. 2; Fig. 5) activated transcription activation of two genes, *sco7700* and *sco7701* (probably forming an operon; Fig. 3), which encode putative enzymes having similarity to nonplant terpene cyclases (*sco7700*) and cyclopropane fatty acid methyltransferases (*sco7701*). These enzymes are involved in the synthesis of terpene or sesquiterpene compounds and would predict role(s) for the air cell-cell signaling in AHF.

In addition to generating spores, aerial hyphae might have evolved in non-motile filamentous bacteria to facilitate oxygen uptake and to increase its availability to the vegetative hyphae of the colony. Oxygen, in fact, may become a limiting factor when cell density reaches high levels inside the colony or when the soil becomes water saturated after an abundant rain, as water would limit oxygen diffusion rates. Thus, the complexity of the environment where *Streptomyces coelicolor* lives, might explain why the genome of an obligate aerobe microorganism possesses three copies of the *narGHJI* operon. This encodes respiratory nitrate reductase (NAR), which is used by micoaerophilic and anaerobic bacteria to respire nitrate and generate energy. Interestingly, it was reported that the *nar2* operon of *S. coelicolor* responded to fluctuations in oxygen levels, thus suggesting that *Streptomyces coelicolor* could generate energy to sustain its basal metabolism through nitrate respiration under oxygen limitation (van Keulen *et al.,* 2005). The results of our genome-wide analyses showed that the expression of the *nar2* operon (as well as of *sco0179*, encoding a putative zinc-containing alcohol dehydrogenase) is developmentally regulated (data not shown), with a rapid decrease in its transcriptional abundance at the onset of AHF in response to SapB (Fig. 2; Fig. 6). This may imply that initiation of aerial growth and, therefore, an higher

oxygen availability for the cells of the mycelium, would generate signal(s) that switch off the expression of genes (*narG2*, *narH2*, and *sco0179*) probably required to ensure a certain energy supply under stress conditions generated by oxygen depletion.

To conclude, our results demonstrated, for the first time, that the biosurfactant activity of a natural amphiphilic compound, like SapB, plays an important role in signaling to the emerging aerial hyphae the activation of a differentiation program needed for the formation of specialized spore-bearing aerial hyphae.

Experimental procedures

Strains and growth conditions

The *ramS null* mutant strain of *Streptomyces coelicolor* used in this study (Nguyen *et al*., 2002) was grown at 30°C on cellophane discs on the complex R2YE medium (Kieser *et al*., 2000).

Aerial hyphae induction of *ramS* mutant with purified SapB

SapB was purified as described by Kodani *et al.,* 2004 and resuspended in 100% DMSO. To have a confluent and homogeneous vegetative mycelium, 2 x 10^6 pregerminated spores (Kieser *et al.,* 2000) from the *ramS null* mutant strain were inoculated on cellophane discs and grown on R2YE plates for 36 h at 30°C. Induction of AHF was performed by overlaying the mycelium with 1.5 μM of SapB (0.5 ml) in incubation buffer (DMSO 50%, 20 mM Tris/HCl pH 7.5). *ramS* mutant overlayed with 0.5 ml of incubation buffer was used as negative control. After the solvent was allowed to evaporate under a flow laminar hood for 10 min at RT (25°C), plates were incubated back at 30°C. AHF was monitored over time by scanning electron microscopy.

RNA isolation

For RNA isolation, mycelium was scraped from the cellophane discs at different times after SapB induction of AHF and immediately treated with RNAprotect bacteria reagent (Qiagen) for 15 min to stabilize the *in vivo* transcript profile. Mycelia were centrifuged at 5,000 x g for 10 min at RT. The pellet was resuspended in 5 ml of RLT™ buffer (Qiagen), sonicated for 15 sec (3x with an output of 80W), and centrifuged at 12,000 x g for 15 min at 4°C to remove cell debris. The supernatant was extracted in phenol/chloroform and then chloroform. The aqueous phase was added to 3 ml of ethanol and loaded onto Rneasy Midi columns (Qiagen). The final steps of RNA purification were carried out according to the protocol in the Rneasy Midi kit (Qiagen). DNA contamination was removed from the RNA samples by DNA-*free*™ kit (Ambion). The quality of the RNA samples was verified by RNA 6000 Nano Assay kit (Agilent Technologies) and by agarose gel electrophoresis. Quantification of RNA was carried out by adsorption at 260 nm.

Microarray analysis and quantitative real time RT-PCR

Microarray analysis of RNA samples was performed as described by Huang *et al*., 2001. For the quantitative real time RT-PCR analyses, first-strand cDNA synthesis was carried out using 2 µg total RNA and SuperScript II (Invitrogen), following the manufacturer's instructions (Cat. No.18064-014). Quantitative real-time PCR of selected genes was performed using the Bio-Rad iCycler™ Real-Time PCR Detection System and iQ™ SYBR Green Supermix Kit (170-8880). 5% of the first-strand reaction was used as DNA template; real-time PCR conditions were as follows: 94°C for 10 min, 40 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. The amount of target cDNA was determined by standard curves and normalized internally to 16S rDNA levels. Relative transcript abundance was calculated as a ratio of normalized values.

Electron microscopy

Low temperature scanning electron microscopy was carried out as previously described (Müller *et al.,* 1991).

Hydropathy pattern analyses

Hydropathy pattern analysis of amino acid sequences was carried out using the Kyte-Doolittle hydropathy algorithms in http://fasta.bioch.virginia.edu/o_fasta/grease.htm

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Concluding Remarks and Future Perspectives

Our studies demonstrated the centrality of the RamR regulon in activating morphological differentiation in *Streptomyces coelicolor*. In addition to transcriptional activation of the *ramCSAB* operon, required for SapB biosynthesis, we showed that RamR activates transcription of a physically distant and distinct group of RamR-dependent genes, *rdgABKR*. RamR and members of RamR regulon may represent an important link between early environmental and physiological regulatory events (checkpoints), mediated by the cascade of *bld* genes, and the activation of the *whi* genes dependent sporulation program that commits aerial hyphae to produce spores. In particular, we showed the importance of the *rdgABKR* operon in such commitment to sporulation, once aerial hyphae have emerged into the air. As a *ΔrdgABKR* mutant was severely delayed in sporulation and differentiate aberrant aerial hyphae, we propose that the *rdgABKR* operon may be part of a transduction network down the sporulation pathway and that its transcriptional dependence on RamR would couple the onset of AHF elicited by SapB biosynthesis (and/or other biosurfactants) with the initiation of the sporulation program. We demonstrated, indeed, that breaching of the air-water interface by means of biosurfactants, like SapB, triggers signaling events that lead to changes in gene expression (*chpABCDFGH*, *rdlAB*, *sco7700*, *sco7701*, *sco2983*, *narG2H2*, and others), coincident with the onset of aerial hyphae formation. Such developmental switch is likely activated by transduction events triggered in the nascent aerial hyphae by yet unidentified signals perceived in the aerial environment.

In addition to commitment to sporulation, members of the *rdgABKR* operon, in particular RdgR, may contribute to AHF by activating, directly or indirectly, production of an extracellular hydrophobic compound(s). This may act synchronously and independently of SapB, providing a secondary and alternative pathway of AHF. Thus members of RamR regulon, temporally regulated by RamR, may function as integrated circuits that connect sequential developmental processes in *Streptomyces coelicolor*.

Clues about the SapB independent and alternative developmental program for AHF may be provided by genome wide analyses of the *ramR* mutant overexpressing the D53E mutant allele of *rdgR* (*ramR/rdgR(D53E*)) relative to the congenic *ramR* mutant strain. Overexpression of *rdgR(D53E)*, in fact, restores AHF and sporulation to the *ramR* mutant (and several other bald mutants) in absence of SapB, representing an ideal genetic background for characterization of the RdgR regulon. Moreover, such

genome-wide analyses may also clarify how and at which regulatory level the genes of the *rdgABK*R operon connect with the *whi* genes of the sporulation cascade.

Purification of wild type RdgR and RdgR(D53E) is, furthermore, needed to confirm a direct role of RdgR in transcriptional regulation of its potential targets (in addition to the genes of the *rdgABKR* operon) and for the biochemical characterization of RdgR.

The *ramR/rdgR(D53E)* (SapB$^-$) strain of *S. coelicolor* may also be suitable for the purification of the predicted hydrophobic morphogenetic compound(s) that, similar to SapB, can elicit AHF. Preliminary data showed, in fact, that application of ethylacetate extracts of the *ramR* mutant strain overexpressing *rdgR(D53E)* can restore AHF to the *ramC* mutant. Improvement of the extraction conditions and franctionation of total extracts, in combination with assays for AHF in recipient bald strains (*ramC* mutant and/or other *bld* mutants), may lead to the isolation and/or characterization of such morphogenetic compound.

Further clues about the developmental program needed for aerial hyphae formation and sporulation in *Streptomyces coelicolor* may also be provided by genetic studies of the members of the “SapB regulon”. Gene disruption and/or overexpression of such genes in several developmental mutants will confirm and clarify whether any role(s) is played in development.

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CURRICULUM VITAE

Name	Salvatore San Paolo
Address	Missionsstrasse 40, 4055 Basel, Switzerland.
Telephone	+41 61 321 86 51
Lab. telephone	+41 61 267 20 71
E-mail	salvatore.san-paolo@unibas.ch
Nationality	Italian
Born on	February the 25th, 1976 in Erice (TP), Italy
Civil state	married

Work experience

April 2006	Postdoc in RNA quality control in *Saccharomyces cerevisiae* and tRNA editing in *Escherichia coli* in the laboratory of Prof. Walter Keller at the division of cell biology of the Biozentrum of Basel University. Collaboration with Dr. Andre' Gerber of ETH, Zurich. Development of X-RIP-chip analysis to identify direct targets of ribonucleoprotein complexes (RNPs) in *Saccharomyces cerevisiae*.
April 2002-March 2006	PhD in development of actinomycetes in the laboratory of Prof. Charles Thompson at the molecular microbiology division of the Biozentrum of Basel University. April 2004: after the moving of Prof. Charles Thompson to the University of British Columbia in Vancouver, I joint the group of Prof. Urs Jenal on the development of *Caulobacter crescentus* in the Biozentrum where I concluded my PhD project. In 2003: collaboration in the laboratory of prof. Cohen at the Medical School of Stanford University. Development of microarray and quantitative RT-PCR analyses of the transcriptome of solid cultures of *S. coelicolor* .

Title of the PhD thesis	"Characterization of regulatory pathways controlling morphological differentiation in *Streptomyces coelicolor"*.
Aims of the PhD thesis	Identification of SapB independent pathways of aerial hyphae formation directly regulated by the response regulator RamR, in the filamentous bacterium *Streptomyces coelicolo*r.
	Investigation of a developmental program needed for aerial hyphae morphogenesis activated at the onset of aerial hyphae formation upon induction with purified SapB.
Teaching activities	Laboratory practice at Basel University: biodiversity of soil samples; quorum sensing in *Vibrio fisheri*; transposon mutagenesis; siRNA and miRNA-mediated gene silencing
March 2002	Degree in Biological Sciences at the "University of Palermo" with a grade of 110/110 cum laude.
Line of study	Molecular Biology and Genetics of Microrganisms.
September 1999-February 2002	Laboratory of prof. Anna Maria Puglia at the University of Palermo. Diploma thesis : "Regulation in the production of the pigmented antibiotic actinorhodin in *Streptomyces lividans"*.
February 1999-June 1999	Trainee in the laboratory of prof. Paul Dyson of the University of Wales in Swansea (UK) :genetic manipulation and transposon mutagenesis of *Streptomyces lividans* for the identification of regulatory elements controlling the biosynthesis of the pigmented antibiotic actinorhodin.
Technical capacity and competences	
	Microbiology and molecular biology techniques
	Transposon mutagenesis in *Streptomyces coelicolor.*
	Genetic manipulation of *Streptomyces* spp..
	Microarray and quantitative RT-PCR analyses
	Biochemestry of hydrophobins and secondary metabolites extracted from the mycelium of solid cultures of *Streptomyces coelicolor.*
	Development of a new method of RNA extraction from solid cultures of *Streptomyces coelicolor*.
	Analysis of the interaction between transcription regulators and their cognate binding sites by EMSA, Dnasel footprinting, S1 mapping experiments.
	Establishment of RIP-chip methodology of *in vivo*
	crosslinked TAP-tagged RNA binding protein

complexes of *S. cerevisiae* .

Deamination assay by one-dimensional TLC.

Publications

Salvatore San Paolo, Jianquang Huang, S.N. Cohen, and Charles J Thompson
rag genes: novel components of the RamR regulon that trigger morphological differentiation in *Streptomyces coelicolor*. *Mol Microbiol* (2006) 61(5), 1167-1186.

Manuscripts in preparation

Salvatore San Paolo, Jianquang Huang, S.N. Cohen, J.M. Willey, and Charles J. Thompson
Genome-wide analyses of *S. coelicolor* revealed that induction of aerial growth by SapB activated a developmental program needed for aerial hyphae biogenesis.

Conferences

Oral presentations:
10th International Congress on the Genetics of Industrial Microorganisms (GIM) on June 24-28, 2006 (Prague).

6° Convegno FISV, Federazione Italiana Scienze della Vita,
30 Sept-3 Oct 2004, Riva del Garda (TN; Italy).

Poster presentations:
2nd ASM coference on prokaryotic development
July 13-16, 2005, Vancouver, BC (Canada).

64th Annual Assembly of the SSM
March 31- April 1, 2005, Genève, (Switzerland)

6° Convegno FISV, Federazione Italiana Scienze della Vita,
30 Sept-3 Oct 2004, Riva del Garda (TN; Italy).

Streptomyces meeting, Münster 2003 (Germany)

Personal capacity and competences

First language Italian

Second language English

Third language French

References

Professor Walter Keller
Department of Cell Biology
Biozentrum
University of Basel
e-mail: walter.keller@unibas.ch

Professor Charles J. Thompson
Department of Microbiology and Immunology

University of British Columbia
Vancouver, B.C., Canada
e-mail: charles.thompson@ubc.ca

Professor Stanley Cohen
Department of Genetics
School of Medicine
Stanford University
Stanford, California (USA)
e-mail: sncohen@stanford.edu