Novel aspects of signaling in Streptomyces development

Wezel, G.P. van; Vijgenboom, E.

Novel Aspects of Signaling in *Streptomyces* Development

GILLES P. VAN WEZEL* AND ERIK VIJGENBOOM

Department of Biochemistry, Leiden Institute of Chemistry
2300RA Leiden, The Netherlands

**Author for correspondence. E-mail: g.wezel@chem.leidenuniv.nl*

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I. Introduction

Actinomycetes have an unusually complex life cycle, many aspects of which are globally similar to those observed in some lower eukaryotes, which makes them particularly interesting for the study of bacterial development and evolution (Chater and Losick, 1997). Their ability to produce a large array of biologically active natural products, including the majority of antibiotics, as well as many agents with other medical and agricultural merits, makes these organisms also highly relevant from an industrial perspective. One of the best-characterized genera among the actinomycetes is *Streptomyces*, the subject of this review, with *Streptomyces coelicolor*, *Streptomyces griseus*, and *Streptomyces lividans* being the most well-studied species. Pioneering work by David Hopwood 40 to 50 years ago established *Streptomyces coelicolor* as the model system for the genus (Chater, 1999; Hopwood, 1999). The latter organism has become the paradigm for the study of *Streptomyces* development (Chater, 1998) and antibiotic production (Bibb, 1996), which was helped by a wealth of mutants and the development of a large genetic toolbox (Kieser *et al.*, 2000). Recently the genome sequences of *S. coelicolor* and *S. avermitilis* were completed, taking *Streptomyces* research into the genomics era (Bentley *et al.*,

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2002; Ikeda *et al.*, 2003). The closely related species *S. lividans* is especially important as an excellent expression host for industrial enzymes. Much more distantly related is the streptomycin producer *S. griseus*, which is of particular genetic interest for two main reasons: the profound and well-studied effect of a signal molecule, the hormone-like A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) on its development and antibiotic production (first described in Khoklov *et al.*, 1967; reviewed in Horinouchi, 2002), and its ability to sporulate in submerged cultures (Kendrick and Ensign, 1983; reviewed in Flärdh and van Wezel, 2003).

On solid media, a germinating spore will produce one or more hyphae, which will grow and branch to form a vegetative mycelium. Exponential growth is achieved by a combination of tip growth and branching, resulting in a complex mycelial network. At this stage, the vegetative hyphae consist of multi-nucleoid syncytial cells separated by occasional cross-walls (Wildermuth, 1970). Then, as colonies develop, an aerial mycelium is produced, with hydrophobic hyphae breaking through the moist surface, erected into the air. This is the start of the reproductive phase, initiated in response to nutrient depletion and the resulting requirement of mobilization. The substrates required for the production of the aerial hyphae are derived from reuse of material such as nucleic acids, proteins, and storage compounds from the vegetative mycelium. Eventually, sporulation-programmed hyphae are formed, producing chains of mono-nucleoid spores, which are released after a poorly understood maturation process. A typical example of colonies of streptomycetes growing on an agar plate is shown in Fig. 1A and a close-up of sporulating aerial hyphae in Fig. 1B.

Mutants that fail to develop an aerial mycelium are called *bld* (bald, reflecting the ''hairless'' phenotype), and mutants that fail to produce mature grey-pigmented spores are called *whi* (white, referring to the production of a nonpigmented fuzzy aerial mycelium). Several reviews have been written on the involvement of *bld* and *whi* genes in the control of *Streptomyces* development and aerial hyphae formation (e.g., Chater, 1998, 2001; Kelemen and Buttner, 1998). In this review we focus on recently discovered genes that play an important role in the two main switches in *Streptomyces* development, namely the *ram* gene cluster (for transition from vegetative to aerial growth) and the *ssgA*-like genes (control of the sporulation process). We also discuss how these and other developmental genes allow streptomycetes to respond to changes in the nutritional state of the microenvironment. Understanding these mechanisms is important for fundamental and applied *Streptomyces* research.

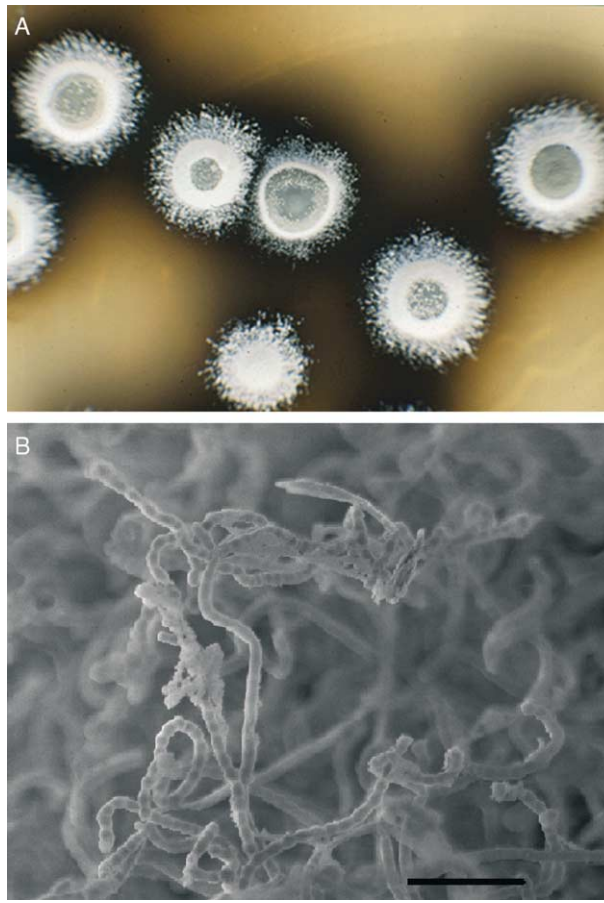

FIG. 1. (A) Sporulating colonies of *Streptomyces ramocissimus*. Clearly visible are aerial hyphae (white outer circle) and spores (grey inner circle); the vegetative mycelium lies below the aerial mycelium and is not visible. The brown pigment secreted by the colonies is melanine. (B) Scanning electron micrograph of sporulating aerial hyphae of *Streptomyces coelicolor*. Photograph courtesy of Dr. H. K. Koerten (Centre for Electron Microscopy, LUMC, Leiden, The Netherlands). Bar = 10 μm.

II. Aspects of Vegetative Growth and Liquid Cultivation of Streptomycetes

Models for mycelial growth have been worked out for filamentous fungi, and particularly the penicillin producer *Penicillium chrysogenum* (Krabben, 1997; Nielsen, 1996; Trinchi, 1971). While at the molecular level the processes are very different in actinomycetes

(prokaryotes) and filamentous fungi (lower eukaryotes), it appears that at the microscopic level these organisms exhibit similar growth forms and hyphal and mycelial growth kinetics (reviewed in Prosser and Tough, 1991). Therefore several of the kinetic models for growth of streptomycetes may be derived from the better-studied fungi (Bushell, 1988). Exponential growth of the mycelium is achieved by a combination of linear (polar) growth, branching, and—particularly in submerged culture—hyphal breakage (Locci, 1980). The frequency of branching is not constant but apparently is dictated by the growth conditions; nutrient-rich conditions favor branching, to optimally profit from the available nutrients in the habitat (typically the soil), whereas under nutrient-depleted conditions branching is reduced and growth is dictated by tip extension, which favors the formation of so-called ''searching hyphae'' (Bushell, 1988). Interestingly, branching and cross-wall formation (which often coincide) markedly reduce hyphal strength (Wardell *et al.*, 2002), a phenomenon supported by the observation that *ftsZ* mutants of *S. coelicolor* are viable and produce unbranched, long and stable hyphae in the absence of cross-walls (McCormick *et al.*, 1994). The relationship between mycelial morphology and stability is particularly relevant for biotechnological applications, because it determines clump size and therefore indirectly also the efficiency of the production process (Bushell, 1988; Wardell *et al.*, 2002).

While development is mostly studied in solid-grown cultures, in the industrial production process large-scale liquid cultures are the reality. Unfortunately, it is difficult to translate morphological principles of one culture type to the other, which is at least in part due to the morphological diversity of liquid-grown mycelium. In batch fermentations, variations as large as three to four orders of magnitude (μm to cm scale) occur. Analysis of erythromycin biosynthesis in *Saccharopolyspora erythraea* showed that production took place only in hyphal fragments with a diameter larger than approximately 90 μm (Martin and Bushell, 1996). Mixing problems with larger mycelial clumps also negatively affect the production process, because an oxygen and nutrient gradient exists from the surface of the mycelial clump to its center, affecting growth and production (Bushell, 1988; Huang and Bungay, 1973). Therefore, better understanding of the factors affecting growth and morphology would obviously be of advantage for biotechnological applications.

Studies in streptomycetes with fluorescently labeled vancomycin or radiolabelled N-acetyl glucosamine (both of which are incorporated into newly synthesized peptidoglycan) revealed that peptidoglycan

biosynthetic activity primarily occurs at hyphal tips and at branching sites (Daniel and Errington, 2003; Gray *et al.*, 1990; Young, 2003). Also, the addition of penicillins results in defects particularly at the apical sites of the hyphae, and less at the lateral walls, which may therefore be regarded as a relatively inert murein polymer. Several of the penicillin-binding proteins and other proteins involved in the synthesis and integrity of the cell wall become recruited to cell wall construction sites. The first clear example of a protein associated with apical growth is DivIVA (Flärdh, 2003). Considering that it is involved in driving (the initiation of) linear extension and that its overexpression results in erratic branching, it is conceivable that another important role for DivIVA is to coordinate the initiation of new branching points. The *Bacillus subtilis* DivIVA homolog plays a direct role in septum-site determination by interacting with the MinCD cell division inhibitor (Edwards and Errington, 1997) and was recently shown to interact with the chromosome segregation machinery to help position the *oriC* region of the chromosome at the cell pole, in preparation for polar division (Thomaides *et al.*, 2001). Streptomycetes lack a homolog of MinC, and the function of the two MinD homolog is unclear, as *minD* disruptants have no obvious phenotype (McCormick and van Wezel, unpublished data). The high frequency of co-occurrence of septa and branches (a feature also seen in filamentous fungi) suggests coordination between cell division and branching, and it is perhaps DivIVA that may play a role in this coordinating process, although there is no evidence that DivIVA directly affects cell division (Flärdh, 2003).

III. The Switch to Development

A. General Considerations

As a result of their mycelial lifestyle, streptomycetes are sessile microorganisms, and in contrast to other bacteria, the mycelium itself cannot migrate to a more favorable environment, such as by chemotaxis. When deprived of nutrients, the mycelium responds by favoring apical (linear) growth over branching (see Section II) and by the onset of morphological differentiation, resulting in the production of exospores. Under nutrient-limiting conditions, lysis of the vegetative hyphae probably provides the nutrients necessary for the construction of the aerial mycelium (Mendez *et al.*, 1985). During this part of the life cycle, several control mechanisms come into play, such as carbon catabolite repression and stringent response, which constitute important sensors of the nutritional state of the environment and have a

repressing or activating effect on the onset of sporulation, respectively. These global regulatory processes and their impact on development are discussed elsewhere (Hodgson, 2000; Ingram *et al.*, 1995; Kamionka *et al.*, 2002; Takano and Bibb, 1994; Ueda *et al.*, 1999).

B. Submerged Development

Typically, ''development'' refers to solid-culture differentiation (vegetative mycelium, aerial mycelium, spores) (Chater, 1972). In liquid culture, several streptomycetes, with *Streptomyces griseus* as the most well-known example, also form submerged spores, produced by sporogenic hyphae at the extremities of liquid-grown vegetative mycelium (recently reviewed in Flärdh and van Wezel, 2003). This process is generally triggered by nutritional shift down, although some streptomycetes also produce submerged spores in nutrient-rich cultures. Comparison of the ultrastructures of submerged spores with surface spores failed to reveal significant structural differences. Perhaps counterintuitively, no differences were found either between the sporogenic hyphae in submerged and solid-grown cultures: both were essentially unbranched and thin-walled (Rueda *et al.*, 2001). The only significant difference was in the sheath, which was thinner and less regularly structured in submerged sporulating hyphae. Some regulatory aspects of submerged sporulation are dealt with in the section on *ssgA* (Section IV.C).

C. Towards an Aerial Mycelium

Early developmental (*bld*) mutants are not only defective in aerial hyphae formation, but also their antibiotic production is strongly affected (either negatively or positively), which links development and secondary metabolism. Most *bld* mutants fail to produce antibiotics, although some are antibiotic overproducers. By definition, all nonessential genes that are required for aerial hyphae formation are *bld* genes, and genes required for any of the developmental stages between the onset of aerial mycelium formation and the production of the spore pigment WhiE are called *whi* genes (Kelemen *et al.*, 1998). There is evidence that several of the *bld* gene products are part of a signaling cascade. This was discovered by extracellular complementation experiments, where *bld* mutants were grown in close proximity to each other, without physical contact (Nodwell *et al.*, 1996; Willey *et al.*, 1991, 1993). A low degree of aerial hyphae formation could be restored by one *bld* mutant to the other, and typically in a unidirectional

manner. A detailed overview of the possible function of the *bld* genes, and their role within the putative signaling cascade, has been provided elsewhere (Kelemen and Buttner, 1998; Nodwell *et al.*, 1999).

The best known of the *bld* mutants is *bldA*, resulting from mutations in the gene for a leucyl tRNA, necessary for the translation of UUA-containing transcripts (Leskiw *et al.*, 1991a,b). Approximately 150 genes of the *S. coelicolor* genome harbor a TTA codon (and are therefore dependent on BldA), many of which are involved in the regulation of development or antibiotic production (Bentley *et al.*, 2002). Interestingly, the failure to obtain *bldA* deletion mutants of *S. coelicolor* M145 suggests that at least one *bldA*-dependent gene is essential for growth in this model strain. Thus, while *bldA* is an important control point on the way to aerial development, at least in M145 it is also required for the translation of nondevelopmental mRNAs.

Recently it was discovered that the activity of several Bld and Whi proteins is not confined to a specific developmental stage. Some of the so-called late *bld* genes—including *bldD*, *bldM*, and *bldN*—are active not only in a stage temporally related to the switch to aerial mycelium formation but are also required much later in the developmental program. For example, *bldM* and *bldN* mutants were originally classified as *whiK* and *whiN*, respectively, as several point mutants obtained from classical screens were blocked in later stages of aerial development. Gene disruption and expression studies later showed that a low level of WhiK and WhiN activity was required for the onset of aerial mycelium formation, after which they were renamed *bldM* and *bldN*, respectively (Bibb and Buttner, 2003; Bibb *et al.*, 2000).

D. Influence of Carbon Sources on Developmental Signaling

The relationship between the nutritional state of the environment and *Streptomyces* development is underlined by the medium-dependence of several of the *bld* mutants, which sporulate on minimal medium agar plates with mannitol but not with glucose (Merrick, 1976; Pope *et al.*, 1996), suggesting a role for glucose repression in *Streptomyces*, mediated through glucose kinase (Angell *et al.*, 1992). Indeed, *glkA* mutant derivatives of *S. coelicolor bldA* mutants do sporulate in the presence of glucose (van Wezel, unpublished data). One *bld* gene that is of particular interest for the link between carbon source-dependent gene regulation and development is *bldB*. BldB null mutants have a bald phenotype on all carbon sources and fail to produce aerial hyphae or antibiotics under any condition (Merrick, 1976). Furthermore, *bldB* mutants are defective in catabolite control

and do not fall in the hierarchy of extracellular complementation exhibited by other *bld* mutants (Nodwell *et al.*, 1999; Pope *et al.*, 1996; Willey *et al.*, 1993). Therefore BldB possibly constitutes a key control point in the switch to development. The *bldB* gene encodes an 11 kDa DNA binding protein that likely functions as a homodimer (Eccleston *et al.*, 2002; Pope *et al.*, 1998). BldB belongs to the family of AbaA-like proteins, with six paralogs on the *S. coelicolor* genome and—similarly to the SsgA-like proteins (Section IV.C)—no clear homologs outside the actinomycetes. Other connections with glucose metabolism have been reported. Ectopic sporulation was observed in an *S. coelicolor* mutant with a 7.4 kb deletion around the *glkA* gene (Kelemen *et al.*, 1995), while an *S. griseus das* mutant produced ectopic spores at regular intervals in the vegetative hyphae, but only on glucose-containing media, providing another example of a carbon source-dependent (conditional) requirement for a developmental gene (Seo *et al.*, 2002). Recently attention has been directed toward the relationship between high-energy tricarboxylic acid (TCA)-cycle intermediates and development. Interruption of aerobic TCA cycle-based metabolism through mutations in citrate synthase or aconitase resulted in irreversible acidification of the medium during growth on glucose, with obvious defects in morphological differentiation and antibiotic biosynthesis. These effects could at least in part be compensated by buffering of the medium (Viollier *et al.*, 2001a,b). This indicates that the outcome of extracellular complementation experiments, such as for the *bld* mutants, should be carefully evaluated, as the signal passed on from one *Streptomyces* strain to another could be the result of an exported (protein) factor, but also of changes in pH or medium composition.

IV. Novel Genes in Development

A. Discovery of New Developmental Genes

The quest for novel developmental genes required different strategies and deployment of new experimental approaches. Originally, the discovery of developmental mutants by selecting/screening typically resulted from random mutagenesis experiments with ultraviolet (UV)-irradiated or chemically treated cells. A large collection of *bld* and *whi* mutants was obtained, which were classified on the basis of morphological characteristics, helped by scanning electron microscopy (Chater, 1972; Hopwood, 1999; Ryding *et al.*, 1999) or transposon mutagenesis (Gehring *et al.*, 2000). Although these studies were extensive, not all the currently known developmental mutants were thus identified.

The complete elucidation of the *S. coelicolor* genome (Bentley *et al.*, 2002), and the concurrent advent of the genomics era to *Streptomyces* research, allowed a directed search for possible or likely developmental genes on the basis of a presumed homology to developmental genes from *Bacillus*, or on the basis of development-dependent expression profiles (Donadio *et al.*, 2002; Hesketh and Chater, 2003; Huang *et al.*, 2001). However, several important new classes of developmental genes were recently identified on the basis of physiological criteria such as the acceleration of aerial mycelium formation in *S. lividans* (*ram* genes, for *r*apid *a*erial *m*ycelium; Ma and Kendall, 1994), restoration of development in the presence of glucose (Seo *et al.*, 2002), or complementation of disturbed submerged sporulation of *S. griseus* mutants (*ssgA*-like genes, for *s*porulation of *S*treptomyces *g*riseus; Kawamoto and Ensign, 1995b). In the following sections we review the complex data generated by several laboratories on two of these examples, namely the *ram/amf* gene clusters and the *ssgA*-like genes.

B. The *ram* and *amf* Gene Clusters

1. *Genetic Organization of the Clusters*

The *ram* (in *S. coelicolor/S. lividans*) and *amf* (in *S. griseus*) gene clusters are important for the transition from vegetative to aerial growth, as well as for early stages of aerial growth. Surprisingly, different screens using complementation of certain phenotypes by genomic libraries all resulted in the identification of the same gene clusters: accelerated aerial hyphae development in wild-type *S. lividans* (Ma and Kendall, 1994); complementation of aerial hyphae development in the A-factor deficient *S. griseus* strain HH1 (Kudo *et al.*, 1995; Ueda *et al.*, 1998); relief of the dependence on increased copper ion levels for development in *S. lividans* (Keijser *et al.*, 2000); and complementation of the *bldJ* mutant (Nguyen *et al.*, 2002). The overall similarity between the *ram* and *amf* genes is not very high, but the gene organization is strictly conserved. Both clusters consist of five genes (Fig. 2): *ramC/amfT* for a serine/threonine (ser/thr) kinase (Hudson *et al.*, 2002), *ramS/amfS* encoding a small peptide, *ramAB/amfAB* encoding an integral membrane ABC transporter, and a fifth oppositely oriented gene *ramR/amfR*, encoding a transcription factor of the two-component regulator family.

2. *Transcriptional Control by RamR*

The *ramR* gene is expressed from a single promoter that is already active in vegetative mycelium and displays its highest activity after approximately 48 hours of growth on solid media, corresponding with

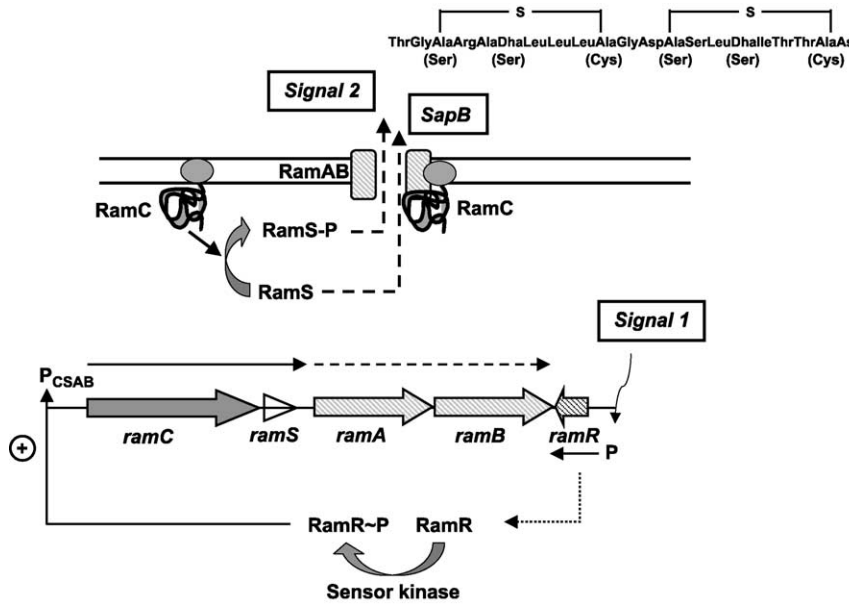

FIG. 2. Model for the transcriptional control of the *ram* cluster by the response regulator RamR, and the function of RamCSAB in the signal transduction. On the basis of sequence analysis, the ABC transporter complex RamAB and the serine/threonine kinase RamC are integrally and C-terminally inserted in the membrane, respectively. In this model, there are two possibilities: (1) RamS is phosphorylated by membrane-bound RamC and subsequently exported by RamAB, and the external RamS then provides a developmental signal (''Signal 2'') to receiver cells; or (2) it is processed and modified to become SapB, a possibility that is strongly supported by the studies of Kodani *et al.* (2004). In the latter case it is not yet clear whether all processing and modification functions are provided by RamC or whether other proteins are involved. A model for the primary structure of SapB is presented in the top right corner (based on Kodani *et al.*, 2004). The RamS residues that are modified in SapB are indicated between brackets below the SapB sequence. The developmental signal that triggers RamR expression (''Signal 1''), and the putative target for RamS signaling, are unknown. Dha, didehydroalanine.

the initiation of aerial hyphae production (Keijser *et al.*, 2002). The same authors showed that *ramR* transcripts are present throughout aerial growth and even during sporulation, although promoter probing assays with *xy/E* as a reporter of transcriptional activity suggested a sudden drop in *ramR* promoter activity upon entry of the aerial growth phase (Nguyen *et al.*, 2002).

In all screens, enhanced expression of RamR/AmfR accelerated development, without the need of the other *ram/amf* genes (Keijser *et al.*, 2000;

Nguyen *et al.*, 2002; Ueda *et al.*, 1993). The important function of RamR was confirmed by mutational experiments, which showed that *S. coelicolor ramR* disruption mutants were severely delayed in development, with sparse and significantly delayed aerial mycelium formation. *S. lividans ramR* mutants were even more delayed in aerial hyphae formation than those of *S. coelicolor*, in line with the idea that the *ram* cluster is more important for the development of *S. lividans* than for the development of *S. coelicolor* (Keijser *et al.*, 2000). Similarly to most other *bld* mutants, *ram* mutants have a conditionally nonsporulating phenotype, with normal development on solid media containing mannitol, and a *bld* phenotype in the presence of glucose. This once more stresses the important role of carbon catabolite control in the decision to switch to development.

Transcriptional analysis showed that expression of *ramC* is dependent on RamR. The target of RamR was identified in studies by O'Connor *et al.* (2002) and Nguyen *et al.* (2002), who showed that RamR has at least one binding site in the *ramC* promoter region. The ability of recombinant RamR to bind *in vitro* to the sequence upstream of the *ramC* promoter demonstrated that phosphorylation of D53, which is essential for the *in vivo* function of RamR, is not required for its DNA binding activity (Nguyen *et al.*, 2002). Similarly, the corresponding amino acid residue in AmfR, D54, was shown to be critical for restoration by AmfR of sporulation to *bld* mutant HH1 of *S. griseus* (Ueda *et al.*, 1993). In *S. griseus*, expression of *amfR* is under control of A-factor through the A-factor-dependent activator protein AdpA (Chater and Horinouchi, 2003; Ohnishi *et al.*, 2002; Ueda *et al.*, 1998). The factors controlling *ramR* expression in *S. coelicolor* and *S. lividans* are unknown, but considering the relatively normal phenotype of A-factor null-mutants (Takano *et al.*, 2001), it is unlikely that AdpA also plays an important role in the control of *ramR*.

3. *RamC Is Essential for Development*

The *ramC* gene did not show up in the screens as an essential gene for the acceleration of aerial hyphae formation. However, constructed *ramC* null mutants were bald (O'Connor *et al.*, 2002) or severely delayed in aerial hyphae production (Nguyen *et al.*, 2002), demonstrating the importance of the gene for development. Analysis of the *ramC* gene product predicted that the protein consists of an N-terminal serine/threonine protein kinase domain and a C-terminal domain with several membrane spanning regions. In line with this prediction, amino acid residues that are invariant and essential in similar kinases were also essential for RamC function, as shown by complementation

experiments in which a *ramC* disruption mutant was transformed with constructs expressing single amino acid substitution mutants of RamC (Hudson *et al.*, 2002). It is attractive to speculate that RamC is associated with the membrane through its C-terminal sequence and functions in close proximity to RamAB (Fig. 2 and Section IV.B.4).

Several studies demonstrated that *ramC* expression occurred at the onset of aerial growth and was dependent on RamR (Keijser *et al.*, 2002; Nguyen *et al.*, 2002; O'Connor *et al.*, 2002). The promoter upstream of *ramC* also directs transcription of *ramS* and *ramAB*, suggesting an operon-like organization. However, the transcription of the *ramCSAB* gene cluster is complex. The *ramAB* genes are transcribed much less frequently because of a transcriptional attenuator between *ramS* and *ramA* (Keijser *et al.*, 2002). Furthermore, the strong accumulation of *ramS* transcripts and the low level of *ramC* transcripts, as detected by Northern hybridization experiments, suggests that after processing of the full-length transcript, the *ramC* mRNA is rapidly degraded, because there is no promoter in the intergenic region between *ramC* and *ramS* (Keijser, 2002).

4. *RamS and RamAB*

What could be the target for the RamC/AmfT kinase activity? The 'S-peptides' (RamS/AmfS) are promising candidates for three reasons. The peptides contain several conserved serine and threonine residues as potential phosphorylation sites (Fig. 3). Ueda *et al.* (2002) showed that the S-peptide has a signaling function and the genes are cotranscribed with the kinase genes. A phosphorylation-dependent function of the S-peptide would fit with a role as signaling molecule. The signal is switched on at a time corresponding to the onset of *ramC* expression, which in turn is activated by RamR, and switched off again as soon as RamC levels drop. Therefore, the signal presumably does not relate to the half-life of RamS. In a hypothetical model, as depicted in Fig. 2, a cascade of events starting with the induction of *ramR*

FIG. 3. Alignment of sequences predicted for the three known "S-peptides." Amino acids shared by at least two proteins are boxed. Residues that form potential target sites for phosphorylation are indicated with asterisks. The accession numbers for the protein sequences are: AAD33774 (RamS *S. lividans*), BAA33539 (AmfS *S. griseus*), and BAC75213 (AmfS *S. avermitilis*). The sequences of *S. coelicolor* and *S. lividans* RamS are identical.

transcription by an unknown signal finally results in the transport of the phosphorylated S-peptide across the membrane. Once outside, the S-peptide will signal the onset of development to neighboring hyphae. Support for this model is provided by several experiments. Disruption of *ramS* in *S. coelicolor* resulted in a severe delay of its aerial hyphae development (Nguyen *et al.*, 2002), while an *amfS* disruption mutant of *S. griseus* had a *bld* phenotype (Ueda *et al.*, 2002). Extracellular complementation of the *amfS* mutant was observed when wild-type *S. griseus* was grown in close proximity. However, neither *amfR* disruption mutants nor *amfAB* disruption mutants were capable of extracellular complementation of the *amfS* mutant. This is supportive of a model in which the extracellular complementing activity is AmfS, which is not produced by the *amfR* mutant and not exported by the *amfAB* mutant (Fig. 2) (Ueda *et al.*, 2002). The observation that the *amfAB* mutant itself shows normal development suggests that sufficient amounts of AmfS are exported (through other transporters) to provide the developmental signal. However, this contradicts the observation that an *amfAB* mutant cannot complement an *amfS* mutant. Therefore, generation of the developmental signal by accumulated AmfS in the cytoplasm, in its native or processed form, is more likely.

The role of RamAB/AmfAB as transporters seems to be crucial for RamS/AmfS activity, although this is not supported by the phenotype of the corresponding disruption mutants. The observation that a *ramB* mutant has a *bld* phenotype (Ma and Kendall, 1994) could not be reproduced by others. The importance of *ramAB* is demonstrated in *S. lividans* by the observation that a triple disruption, Δ*ramABR*, has a *bld* phenotype, while the Δ*ramR* mutant in the end does produce some aerial hyphae (Keijser *et al.*, 2000). Interestingly, a synthetic full-length AmfS peptide was not capable of inducing aerial hyphae formation in the *amfS* mutant, but a synthetic C-terminal octapeptide did induce aerial growth in the mutant (Ueda *et al.*, 2002).

5. *Are RamS and SapB the Same Protein?*

Another small peptide implicated in the onset of aerial hyphae formation is SapB (Willey *et al.*, 1991), which has been suggested to be identical to RamS (Chater and Horinouchi, 2003). This hypothesis was supported by the observation that SapB levels are significantly higher in strains carrying multiple copies of *ramR* or *ramSABR* (Keijser *et al.*, 2002; Nguyen *et al.*, 2002). The N-terminal amino acid sequence of SapB, TG(S/G)RR, is 4/5 identical to residues 22 to 26 of RamS (Keijser *et al.*, 2002; Willey *et al.*, 1991). Assuming that SapB consists of the C-terminal 21 amino acids of RamS, a mass of 2099 Da would be

expected. However, the reported mass of SapB is 2027 Da (Keijser *et al.*, 2003; Willey *et al.*, 1991,). In an elegant new study by Kodani *et al.* (2004), it was shown that the mass of 2027 Da is in agreement with a posttranslational modified peptide consisting of the C-terminal 21 amino acids of RamS, with four out of five serine residues dehydrated resulting in didehydroalanine residues. Two of these residues then react with the two cysteine residues, producing two eight-membered rings with thioether lanthionine bridges (Fig. 2). The latter modifications are suggested to be introduced by the C-terminal domain of RamC that shows significant similarity to lantibiotic modifying enzymes. Whether the N-terminal domain of RamC, having similarity to ser/thr kinases, plays a role in the RamS processing remains to be elucidated. Another question that remains relates to the initial observation that SapB reacts with Schiff's reagent (Willey *et al.*, 1991), indicating the presence of a sugar residue or another molecule with vicinal hydroxyl groups, which is not explained by the current structure.

C. Novel Regulators of Sporulation: The SsgA-Like Proteins (SALPs)

1. *Occurrence of SALPs*

Another novel family of developmental regulators first identified in streptomycetes, and later also in other *Actinomyces* species such as *Thermobifida* and *Streptoverticillium*, is that of the SsgA-like proteins (SALPs; Pfam PF04686). The surprising finding that enhanced expression of SsgA directly stimulates sporulation-specific cell division indicates that SsgA is an important control point in the onset of sporulation. This is supported indirectly by phylogenetic evidence, because SALPs are apparently unique to sporulating actinomycetes and are absent from the nonsporulating actinomycetes *Corynebacterium glutamicum, Mycobacterium leprae*, and *Mycobacterium tuberculosis* (van Wezel *et al.*, 2000a).

The recently completed genome sequences of *S. avermitilis* and *S. coelicolor* revealed six and seven *ssgA*-like genes, respectively (Bentley *et al.*, 2002; Ikeda *et al.*, 2003). The genes encode relatively small (130–140 aa) proteins, which share 30–50% amino acid identity (Keijser *et al.*, 2003; van Wezel *et al.*, 2000a). Homologs of *S. coelicolor ssgA* (Sco3926), *ssgB* (Sco1541), *ssgD* (Sco7622), and *ssgE* (Sco3158) are found on the *S. avermitilis* genome (Sav3926, 6810, 1687, and 3605, respectively), with high conservation in these otherwise distantly related species: The *ssgB* gene products differ in only one amino acid residue. The highest conservation is found in two sections

of the proteins, corresponding to amino acid residues 13–30 and 40–65 of SsgA. In total, 20 amino acid residues (approximately 15% of the protein) are fully conserved among all 19 SALP proteins identified so far. However, there are no sequences in these proteins that resemble known functional motifs.

2. *SsgA Triggers Sporulation-Specific Cell Division*

Studies on *ssgA* strongly suggest that it is an activator of sporulation-specific cell division. *ssgA* was originally identified as a suppressor of the hyper-sporulating mutant SY1 of *Streptomyces griseus* B2682 and shown to be involved in the regulation of submerged sporulation (Kawamoto and Ensign, 1995a; Kawamoto *et al.*, 1997). The gene is of particular interest for both applied and fundamental aspects of *Streptomyces* research, as its expression level apparently controls morphology and development. Increased expression of SsgA alters the phenotype of liquid-grown mycelium of *S. coelicolor*, which normally forms large clumps but produces open mycelial structures (so-called mycelial mats) when expression is increased and shows fragmentation and submerged sporulation at high levels (van Wezel *et al.*, 2000a,b). At these high expression levels, thick and amorphous septa are formed at regular intervals, forming spore-like compartments (Fig. 4). The stimulation by SsgA is apparently specific to sporulation-specific cell division, as *ssgA* mutants are defective in sporulation but form normal vegetative septa (Jiang and Kendrick, 2000; van Wezel *et al.*, 2000a). Interestingly, *ssgA* mutants produce viable spores on mannitol-containing media, making it the only known ''conditional'' *whi* mutant.

Transcriptional analysis showed that *ssgA* is transcribed from two developmentally regulated promoters in both *S. coelicolor* and in *S. griseus* (Traag *et al.*, 2004; Yamazaki *et al.*, 2003). One of these promoters is species-specific, suggesting that *ssgA* is regulated differently in these two organisms. This is indeed the case. In *S. griseus*, transcriptional activation of *ssgA* (further designated $ssgA_{sg}$ to discriminate it from *ssgA* from *S. coelicolor*, referred to as $ssgA_{sc}$) is dependent on the y-butyrolactone A-factor (Horinouchi, 2002; Horinouchi and Beppu, 1995) and involves at least three different regulatory proteins. First, its transcription requires activation by AdpA, a central regulator of the A-factor pathway, which was shown to bind to three distinct sites upstream of *ssgA* (Yamazaki *et al.*, 2003). The same authors showed that $ssgA_{sg}$ transcription is (probably indirectly) dependent on AdsA, the homolog of the developmental σ-factor σ^{BldN} of *S. coelicolor*. Moreover, $ssgA_{sg}$ is most likely activated by the upstream located *ssfR* (*ssgR* in *S. coelicolor*), which encodes an IcIR-type transcriptional

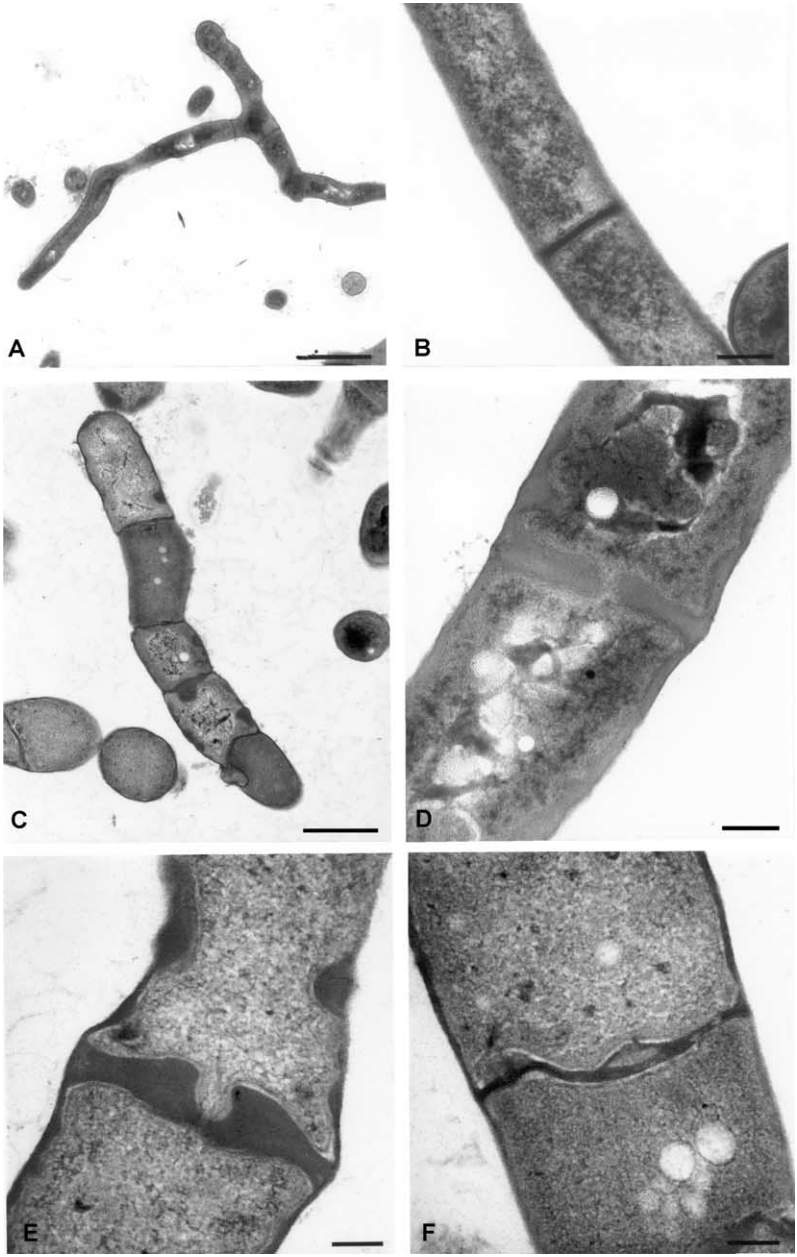

FIG. 4. Effect of enhanced expression of SsgA on the morphology of submerged hyphae and on septation of *S. coelicolor* A3(2). Transmission electron micrographs show

regulator. Although *ssgA* is still transcribed in an *ssfR* mutant, one of its promoters is strongly down-regulated.

Interestingly, while transcription of *ssgA* is fully dependent on A-factor in *S. griseus*, it most probably is not in *S. coelicolor*. Here, the upstream located *ssgR* gene is essential for its activation, and both promoters are silent in an *ssgR* mutant (Traag *et al.*, 2004). Considering the dominant role of SsgA in triggering *Streptomyces* cell division, this could explain why A-factor plays such an important role in development of *S. griseus* but not in *S. coelicolor*. The apparent requirement for tight control of *ssgA* transcription may be necessitated by the profound changes in *Streptomyces* morphology brought about by fluctuations in the *ssgA* expression level.

While transcription of *ssgA* and *ssgR* is strongly up-regulated during the onset of sporulation in *S. coelicolor*, it is not significantly affected in any of the so-called early *whi* mutants of *S. coelicolor* (*whiA, whiB, whiG, whiH, whiI*, and *whiJ*) (Traag *et al.*, 2004). This places these genes outside the generally accepted regulatory cascade leading to solid-culture sporulation. This is the first clear example of a sporulation gene that is expressed in a *whi*-independent manner. The physiological reason for this may be to provide a way to bypass the *whi* cascade under conditions where aerial hyphae formation is not desired, such as during ectopic or submerged sporulation. It is unclear what morphological changes occur during liquid-culture differentiation, but the fact that it does not require an aerial mycelium implies that there are two routes towards sporulation: one via the traditional *whi* cascade and one via the *whi*-independent route. SsgA is essential for submerged sporulation, and if *ssgA* transcription would be fully dependent on (some of) the *whi* genes, this process would probably be impossible because it is likely that several of the *whi* genes are not expressed under submerged conditions. This working hypothesis requires further testing in *S. griseus*.

3. ssgB *Is Essential for Sporulation*

Expression profiling studies with DNA microarrays by the Cohen laboratory revealed two other SALPs as possibly developmentally regulated, namely *ssgB* and *ssgD* (Huang *et al.*, 2001). Of these, *ssgB* has

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S. coelicolor M145 (A–B) and *S. coelicolor* GSA2 overexpressing SsgA (C–F). (A) image showing vegetative hyphae and cross-walls; (B) Magnification of wild-type vegetative septum; (C) Image showing submerged hypha forming pre-spore-like compartments as the result of the overexpression of SsgA; (D–F) examples of abnormal septa in GSA2. Magnifications: (A) and (C), bar = 1 μm; (B, D–F): bar = 0.2 μm. Figure reproduced from van Wezel *et al.*, 2000a, with permission from the American Society for Microbiology.

been subject of more detailed transcriptional and mutational analysis. Like SsgA, SsgB is involved in the regulation of *Streptomyces* development but acts in an earlier phase of the sporulation process. Detailed studies with confocal fluorescence microscopy and electron microscopy showed that *ssgB* deletion mutants fail to produce sporulation septa, and genome segregation and condensation were not observed. In contrast to *ssgA* mutants, the nonsporulating phenotype could not be rescued by growth on mannitol-containing media. These *ssgB* mutants produce colonies that are larger than those of the parental (wild-type) strain (Keijser *et al.*, 2003), possibly linking SsgB to the process of growth cessation that occurs prior to sporulation-specific cell division (Chater, 1989, 2001; Flärdh *et al.*, 1999).

The developmental role of *ssgB* is underlined by the observation that transcription of *ssgB* coincides with aerial mycelium formation and depends on the developmental σ^{H} (Kormanec and Sevcikova, 2002), a σ factor that itself is developmentally controlled at the transcriptional and post-translational level and plays a role in stress responses (Kelemen *et al.*, 2001; Viollier *et al.*, 2003). However, while *sigH* mutants are still able to produce spores, *ssgB* mutants are not (Keijser *et al.*, 2003). This suggests that *ssgB* is transcribed by at least one other σ factor, active earlier in the developmental program.

Interestingly, the BldD protein is involved in the repression of the *sigH*p2 promoter, and therefore indirectly of *ssgB*. BldD is a repressor protein that becomes active at the end of the *bld* signaling cascade and controls transcription of the developmental σ factor genes *whiG* and *bldN/whiN*. These genes play crucial roles during several stages of aerial mycelium formation. This is a clear example of links that exist between the regulation of the switches to aerial mycelium formation (by the *bld* genes) and to sporulation (by *whi* genes).

4. *What Is the Function of the SALPs?*

The mode of action of the SsgA-like proteins is as yet unknown. The relatively highly conserved region corresponding approximately to amino acid residues 20–70 possibly lends a common function to the SALPs, such as interaction with the same protein or protein complex, while the highly variable N- and C-terminal parts are likely to provide functional specificity to the individual SALP proteins. Recent data suggest that they are expressed during distinct phases in the *Streptomyces* life cycle and may play a role in the coordination of cell division and DNA segregation (Noens, Koerten, and van Wezel, unpublished data). In accordance with this idea, when the amino acid sequence most conserved among SALPs is used in a database screen, the only

non-SALP hit is MukB, a protein also involved in DNA segregation in bacteria (van Wezel and Vijgenboom, unpublished). Structural and physiological studies are required to elucidate the role of this interesting new family of proteins.

V. Concluding Remarks

In recent years several new developmental genes have surfaced. The discovery and characterization of genes such as *ram/amf* and the members of the family of *ssgA*-like genes have shed new light on the complex morphological development in *Streptomyces*. Also, the first insights were provided into the relationship between carbon metabolism and development. However, the picture is still far from complete. Important missing links are the signaling molecules, the signal receptors, and the signal transducers, postulated for connecting the individual parts of the developmental machinery, and the exact timing of their expression. Designing new approaches to disclose the identity of many more components of the developmental system is the challenge for the years to come. Helped by the recent elucidation of the complete genome sequences of *S. coelicolor* and *S. avermitilis*, and with that of many others soon to follow, significant research efforts employing functional genomics, classical biochemistry, and protein chemistry are needed to identify and characterize the components involved in the morphological development of streptomycetes.

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