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Keijser, B.J.F.; Wezel, G.P. van; Canters, G.W.; Vijgenboom, E.

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## Developmental Regulation of the *Streptomyces lividans* *ram* Genes: Involvement of RamR in Regulation of the *ramCSAB* Operon

Bart J. F. Keijser, Gilles P. van Wezel, Gerard W. Canters, and Erik Vijgenboom\*

Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, 2300 RA Leiden, The Netherlands

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**Streptomyces** are filamentous soil bacteria that produce spores through a complex process of morphological differentiation. The *ram* cluster plays an important part during the development. The *ram* genes encode a membrane-bound kinase (RamC), a small protein (RamS), components of an ABC transporter (RamAB), and a response regulator (RamR). While the introduction of an extra copy of the *ram* cluster accelerates development in *Streptomyces lividans*, *ramABR* disruption mutants are unable to produce aerial hyphae and spores. The developmental regulation of *ram* gene transcription was analyzed. Transcription of the *ram* genes occurred only on solid rich media and not on minimal media. The *ramR* gene is transcribed from a single promoter during all growth stages, with the highest levels during aerial growth. The *ramCSAB* genes comprise one operon and are transcribed from one principal promoter, P1, directly upstream of *ramC*. Transcription of *ramCSAB* was already observed during vegetative growth, but was strongly upregulated upon initiation of formation of aerial hyphae and was decreased during late stages of development. A large inverted repeat located downstream of *ramS* terminated the majority of transcripts. The introduction of *ramR* on a multicopy vector in *S. lividans* strongly induced P1 activity, while disruption of this regulator eliminated all P1 promoter activity. This shows that *ramR* is a crucial activator of *ramCSAB* transcription. Importantly, in *bldA*, *bldB*, *bldD*, or *bldH* mutants, *ramR* and *ramCSAB* are not transcribed, while *ram* gene transcription was observed in the earliest *whi* mutant, *whiG*. This indicates that the transcription of the *ram* genes marks the transition from vegetative to aerial growth.

Many unicellular bacteria can temporarily adopt a multicellular organization to perform specific tasks. Examples of this include swarming, in which numerous cells aggregate and move collectively over a surface, and myxospore formation, which is preceded by aggregation resulting in the formation of a sacculus (stalk of fruiting body) allowing the subsequent development of resistant cells (myxospores) (31). Different from most prokaryotes, the gram-positive soil bacterium *Streptomyces* mainly exists as a multicellular organism. Streptomycetes rely on a process of spore formation for propagation and survival. The life cycle of these organisms resembles that of filamentous fungi. After germination of a spore, a network of branched filamentous vegetative hyphae is produced. At late vegetative growth, secondary metabolism is switched on and reproductive growth is initiated. One of the first steps in reproductive growth is the production of hydrophobic aerial hyphae that erect themselves out of the plane of the agar. The initiation of formation of aerial hyphae is believed to be dependent on metabolic status, stress, and a cascade of extracellular signaling molecules (17, 41). Aerial hyphae undergo several transformation steps ultimately leading to the formation of unigenomic spores at the hyphal tips (5, 9). Elemental steps in the process of morphological development have been elucidated by the characterization of developmental mutants. Two classes of developmental mutants are known: those that are unable to produce aerial hyphae (*bld* mutants) and those that

are unable to produce gray mature spores (*whi* mutants) (3, 4, 11, 15, 24, 27, 29, 41). As the result of genetic analysis of *whi* and *bld* mutants, a model of the genetic regulation and interplay of the developmental genes during this process is now emerging (6, 9).

Developmental genes have also been identified because of their ability to stimulate or delay the process of morphological development in mutant or wild-type strains. For example *craA*, encoding a carbon source-dependent repressor molecule, was identified by the ability to block morphological development of *Streptomyces griseus* when introduced on a multicopy vector (34). Conversely, Ueda and coworkers identified a cluster of genes (the *amf* cluster) that restored the development of an A-factor-deficient developmental mutant of *S. griseus* (HH1) when overexpressed (36). In a similar fashion, the *Streptomyces coelicolor ram* cluster was identified after it was found to stimulate formation of aerial hyphae of *S. lividans* when introduced on a low-copy-number vector (22). The *amf* and the *ram* cluster appear to be homologous, although the level of DNA sequence identity between both clusters is not as high as one would expect from orthologues. However, the overall architectures of the open reading frames (ORFs) are fully identical, and the proteins encoded are highly similar. Both gene clusters consist of a gene encoding a serine threonine kinase (*ramC* or *amfT*), a gene encoding a small protein with unknown function (*ramS* or *amfS*), two genes that are translationally coupled encoding components of an ABC transporter (*ramA* and *ramB* or *amfB* and *amfA*), and a response regulator (*ramR* or *amfR*) oriented in the opposite direction. The molecular basis for the accelerated development of *Streptomyces lividans* complemented with the *S. coelicolor ram* genes is not known. As

\* Corresponding author. Mailing address: Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands. Phone: (31) 71 5274278. Fax: (31) 71 5274349. E-mail: vijgenbo@chem.leidenuniv.nl.

TABLE 1. Sequences of the PCR primers used to produce promoter-probe fragments<sup>a</sup>

Primer	5'→3' sequence	Description
DSR1	GCC <b>GGA TCC</b> CAT GGC CTC TTC CTT GGG	Reverse primer pPS clones
DSF1	GCC <b>GAA TTC</b> GAC GGG CTC GCC GGA CTC GCC	Forward primer pPS1
CIN2	GCC <b>GAA TTC</b> GGT TCG TGG CGC CTC CCG ACC	Forward primer pPS2
CIN	GCC <b>GAA TTC</b> GCT GCG CGG TCC GGT CTT CCG	Forward primer pPS3
S1F	CGT <b>GAA TTC</b> GAC GCG CCC GCC GGA CTG CCG	Forward primer pPS4
RCUP	GGC ACG TAC ACG CAG GTG CCG	Forward primer pPSt
PRAMA4	GCC <b>GGA TCC</b> GAG AAC CTC CGT CAG TCG AGC GC	Reverse primer pPA1
PRAMR1	GCC <b>GGA TCC</b> CCC ACC CCT TGT CAG CGG CGG CC	Forward primer pPR1
PRAMR2	GCC <b>GAA TTC</b> GGA GCC GAT CGC GCG GTG CC	Reverse primer pPR1

<sup>a</sup> Shown are the sequences of PCR primers used for generation of the DNA fragments cloned into vector pIJ2587 and used for promoter-probing experiments. The restriction sites that were introduced for cloning purposes are indicated in boldface.

observed with the *S. coelicolor ram* cluster, the *S. lividans ram* cluster (which is 99.7% identical to the *S. coelicolor ram* cluster) also accelerated the development of this strain. This indicates that the stimulation of the development in this strain is due to a multicopy effect rather than to complementation of a "defective" *S. lividans ram* cluster. Transcription analysis revealed that *ramAB* transcript levels are extremely low in both *S. coelicolor* and *S. lividans*. *S. lividans* transformants carrying an extra copy of the full *ram* cluster displayed an elevated *ramAB* transcript level (16). The importance of the *ram* and *amf* genes has been demonstrated by the phenotypes observed with disruption mutants. The disruption of *amfR* in *S. griseus* results in a fully *blt* phenotype (35). The disruption of *ramABR* in *S. lividans* also resulted in a *blt* phenotype, while *ramR* disruption mutants display a delayed development (16).

To further examine the role of the *ram* genes during morphological differentiation, the transcription of the *S. lividans ram* genes was analyzed during growth and development. Furthermore, the effect of *ramR* copy number on *ramCSAB* transcription was examined. To relate *ram* gene transcription to a characteristic developmental stage, transcription in various developmental mutants was examined. The results demonstrated the intricate relationship between *ram* gene expression and the onset of production of aerial hyphae.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *Streptomyces* strains were routinely grown on the rich solid medium R2YE or the minimal medium SMM (18). For growth in liquid medium, YEME supplemented with 1% glycine and 1% MgSO<sub>4</sub> or TSBS (3% [wt/vol] tryptone soya broth with 10% [wt/vol] sucrose) was used (18). The streptomycetes strains used in this study are *S. lividans* 1326, *S. coelicolor* A3(2) M145, M512 (M145 *ΔredD actII orfIV*) (10), bldA39 (J1700) (20), bldD53 (J774) (24), and bldH109 (WC109) (3), as well as a *whiG::hyg* (J2400) strain (9).

*Escherichia coli* JM109, which was used as a host for pTZ19R clones, was cultivated at 37°C in Luria-Bertani (LB) medium or on LB agar plates (30). The modification-deficient *E. coli* strain ET12567 was used for the generation of unmethylated DNA, which was used for the transformation of *S. coelicolor* strains (23). Antibiotics (ampicillin, apramycin, and hygromycin) were purchased from Ducheva (Haarlem, The Netherlands). Thiostrepton was a kind gift of Bristol-Meyers Squibb.

**In vitro DNA manipulations.** DNA manipulation and transformation of *E. coli* were performed according to standard procedures (30). Isolation of *Streptomyces* plasmid DNA and polyethylene glycol-assisted transformation of *Streptomyces* protoplasts were performed according to protocols described in reference 18.

PCR was performed in a minicycler (MJ Research, Watertown, Mass.) with *Pfu* polymerase (Stratagene, La Jolla, Calif.) and the buffer provided by the supplier in the presence of 5% (vol/vol) dimethyl sulfoxide, 25 pmol of each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), and 10 ng of tem-

plate. The amplification was performed in 30 cycles consisting of 60 s at 94°C, 60 s at 54°C, and 60 s at 72°C.

**In vivo promoter-probe experiments.** The low-copy-number plasmid pIJ2587 (approximately 5 to 10 copies per genome) was used to detect promoter activity of the various *ram* DNA fragments (38). This plasmid contains the promoterless undecylprodigiosin biosynthesis activator gene *redD*, which allowed promoter activity in cloned fragments to be visualized by the red pigment production induced in the pigmentation-deficient strain *S. coelicolor* strain M512 (*ΔredD actII orfIV*) (10). DNA fragments of the upstream regions of *ramC*, *ramA*, and *ramS* (shown in Fig. 3A) as well as *ramR* were amplified by PCR with the primers listed in Table 1. During amplification, an *EcoRI* restriction site was created at the 5' end and a *BamHI* site was created at the 3' end, which allowed directional cloning into vector pIJ2587. For construction of pPSt, the unique *EcoRI* restriction site present upstream of the *ramC* start codon was used for cloning. Following introduction into *S. coelicolor* M512, transformants were analyzed by standard plasmid preparation and restriction analysis. All promoter-probing transformants produced normal levels of aerial hyphae and spores. Quantitative analysis of undecylprodigiosin production was performed by harvesting mycelium of the M512 transformants grown on cellophane-overlaid R2YE plates at various moments during growth followed by repeated extraction of 100 mg of mycelium with 500 μl of methanol and measurement of the absorption at 533 nm. From each transformant, undecylprodigiosin was extracted from two independent cultures that both gave comparable results (listed in Table 2).

**RNA isolation.** *Streptomyces* mycelium was harvested at various points during growth on R2YE plates that were overlaid with polycarbonate track-etch (PCTE) membranes (0.2-μm pore size) (Osmotics, Livermore, Calif.). Total RNA was isolated by the hot phenol-sodium dodecyl sulfate extraction method as described in reference 18 followed by DNase I treatment. The total RNA concentration for each sample was determined spectrophotometrically. The quality of the isolated RNA was analyzed on a 1.5% denaturing TAE agarose-formaldehyde gel (30).

**Nuclease S1 mapping.** For each nuclease S1 protection assay, 0.02 pmol (5 × 10<sup>4</sup> Cerenkov counts min<sup>-1</sup>) of labeled probe was coprecipitated with 40 μg of RNA and resolubilized in Na-trichloroacetic acid buffer (26). After denaturation of the RNA-probe mixture at 72°C for 15 min, samples were allowed to cool down overnight to a final temperature of 45°C. Subsequently, samples were diluted in 300 μl of ice-cold S1 buffer (8) containing 100 U of S1 nuclease and chilled on ice. Digestions were performed at 37°C for 45 min, after which the samples were precipitated with 0.1 volume of 3 M sodium acetate (pH 6) and 2 volumes of ethanol. After centrifugation, the pellets were dissolved in standard sequencing gel loading dye, heat denatured, and electrophoresed on denaturing 6% (wt/vol) polyacrylamide sequencing gels. The DNA probe that was used to detect the transcription start upstream of *ramC* (*pcp1*) was amplified with primer RCRV (5'-CGT AGA AGT ACG GGT CCG CGT CGC-3'), which was designed based on the sequence approximately 120 bp downstream of the annotated translation start site of *ramC*, and the M13 reverse sequencing primer (5'-AAC AGC TAT GAC CAT G-3'). As a template we used pTZ19R with an *EcoRI-BamHI* subclone of the *ram* cluster encompassing *ramC*, *ramS*, *ramA*, and part of *ramB* (pBK105). The 254-bp probe contained a 64-bp nonhomologous extension that allowed discrimination of signals originating from reannealing of the probe and full-length protection of the probe by the messenger. Similarly, the DNA probe (*pcp2*) used to identify the second transcription start site that lies within the *ramC* ORF was amplified with primer SUP (5'-GTG CGC CAG CCC TCG TCG ACC-3') and the M13 sequencing primer (5'-GTA AAA CGA CGG

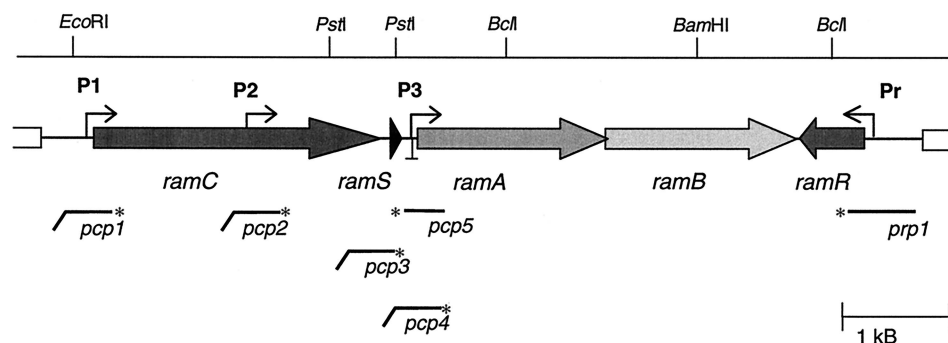


FIG. 1. Genomic structure of the *ram* cluster. The arrows indicate the *ram* genes. *ramC* encodes a membrane-bound serine/threonine kinase, *ramS* encodes a small protein with unknown function, *ramA* and *ramB* are translationally coupled and encode components of an ABC transporter, and *ramR* encodes a response regulator. P1, P2, and P3 mark the promoter regions involved in transcription of the *ramCSAB* genes, while Pr marks the *ramR* promoter. The symbol  $\perp$  downstream of *ramS* marks the position of a transcriptional terminator. Below the map are indicated the DNA probes used in high-resolution nuclease S1 protection assays. The asterisks represent the  $^{32}\text{P}$ -labeled end. The horizontal lines indicate homologous regions, while the diagonal parts indicate the nonhomologous parts of the probe (usually derived from cloning vector pTZ19R).

CCA GT-3'), with pBK118 as a template. Plasmid pBK118 contains an *SphI*-*BclI* fragment of the *ram* cluster encompassing the region between the 3' end of *ramC* and the 5' end of *ramA* cloned into pTZ18R. For detection of transcription into *ramS*, a DNA probe (*pcp3*) was amplified with pBK156 as a template and primer RSRV (5'-GTG ATG CTC AGG CTG AGG CTG CTG TCG-3') and the M13 reverse primer. Plasmid pBK156 contains the *EcoRI*-*BamHI* insert of pPA1 encompassing parts of *ramC*, *ramS*, and *ramA* cloned into pTZ19R. For the detection of *ramR* transcription, a 500-bp probe was generated by PCR with primers S1RF (5'-GAC TGC GAG GAC ACG TCC AGC-3') and RPR2 (5'-GGA GCC GAT CGC GCG GTG CC-3'), with pBK103 containing the *BamHI*-*SphI* fragment of *ramB* and *ramR* as a template. The *ramAB* S1 probe (*pcp4*), which was used to detect the *ramAB* transcripts, was generated by PCR with *pramAB* (5'-CGG CCG CCG CGA CCG AGC AC-3'), which was designed based on the sequence approximately 80 bp downstream of the putative translation start site of *RamA*, and the M13 reverse sequencing primer (5'-AAC AGC TAT GAC CAT G-3'). As a template, we used a *PstI*-*BclI* subclone containing parts of *ramS* and *ramA*. Probes were labeled at the 5' end with [ $\gamma$ - $^{32}\text{P}$ ]ATP and T4 polynucleotide kinase. To enable the interpretation of the signals, dephosphorylated *HaeII*-digested pBR322 was labeled with [ $\gamma$ - $^{32}\text{P}$ ]ATP and run alongside the reaction products. For an accurate determination of the transcription start sites, the S1 samples were accompanied by a sequence ladder on the acrylamide gel. Sequencing was performed with the T7 DNA polymerase sequencing kit (Amersham) according to the manufacturer's recommendations by using PCR probes used for the protection assays as a template. To detect the 3' end of *ramCS* transcripts, probe *pcp4* was digested at a unique *NcoI* site located within *ramS* (Fig. 5B). After isolation of the 270-bp fragment, the 5' overhang of the *NcoI* site was filled in with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by using Klenow fragment in the presence of 0.1 mM dATP, dGTP, and dTTP and 1 $\times$  OnePhorAll buffer (Pharmacia).

## RESULTS

***ramR* is transcribed from a single promoter during all stages of growth on solid media.** The transcription of *ramR* in *S. lividans* was analyzed during morphological development by nuclease S1 protection assays. RNA derived from *S. lividans* grown on PCTE-overlaid R2YE harvested at different time points was hybridized with probe *prp1*, which encompassed the N-terminal part of *ramR* and upstream regions (Fig. 1). After processing, the samples were run on a denaturing polyacrylamide gel electrophoresis gel alongside a sequence ladder produced with the oligonucleotide S1RF (see Materials and Methods) (Fig. 2A). A band corresponding to *ramR* transcripts was detected in all RNA samples, its intensity increasing during vegetative growth to a maximum at the onset of production of aerial hyphae. The 5' end of the transcript corresponds to

nucleotide position  $-66$  relative to the *ramR* translational start site (Fig. 2B). Promoter-probing experiments revealed promoter activity within this region, indicating that the signals of the S1 protection assays represent de novo transcription (data not shown). Analysis of the DNA sequence for possible promoter consensus sequences revealed the sequence GCACAT around  $-10$ , while an apparent  $-35$  sequence is missing. Therefore,  $P_{ramR}$  does not fall into the class of  $\sigma^{70}$ -like promoters.

**In vivo promoter probing of the *S. coelicolor ramCSAB* gene cluster.** Since it has been established that transcription of *ramAB* is coupled to that of *ramS* and the region between *ramC* and *ramS* is relatively small (37 bp) (Fig. 5B), it seems likely that *ramC*, *ramS*, and *ramAB* are transcriptionally coupled, constituting a single operon (16). To analyze the transcriptional regulation of the putative *ramCSAB* operon, promoter-probe experiments were carried out. For this purpose, *S. coelicolor* M512 (M145  $\Delta redD actII orfIV$ ) was transformed with several derivatives of vector pIJ2587, in which various DNA fragments of the *ramCSAB* operon were cloned (Fig. 3A). The promoter activity of DNA fragments cloned into pIJ2587 can be assessed visually by the undecylprodigiosin (Red) production elicited in the host strain M512.

The largest fragment, pPSt, encompassing *ramC* and flanking sequences, strongly stimulated red pigment formation in *S. coelicolor* M512 on R2YE, indicative of the presence of at least one strong promoter (Fig. 3B). A similar result was obtained with the *EcoRI*-*KpnI* fragment of pPSt containing the *ramC* upstream region and the first 100 bases of *ramC*, hereafter referred to as P1 (Fig. 3). In both transformants, undecylprodigiosin became apparent after 30 h of growth on R2YE (Table 2). Fragments in which the *ramC* upstream region as well as portions of *ramC* were deleted, but the sequence between the *NotI* and *PstI* sites within *ramC* was retained (we hereafter refer to this latter region as P2), all resulted in significantly lower, but still readily detectable, levels of Red production that coincided with production of aerial hyphae (Fig. 3 and Table 2). The region of approximately 250 bp directly upstream of *ramS* (pPS1) did not elicit any Red production. A construct encompassing 400 bp upstream of *ramA* (pPA1) did result in

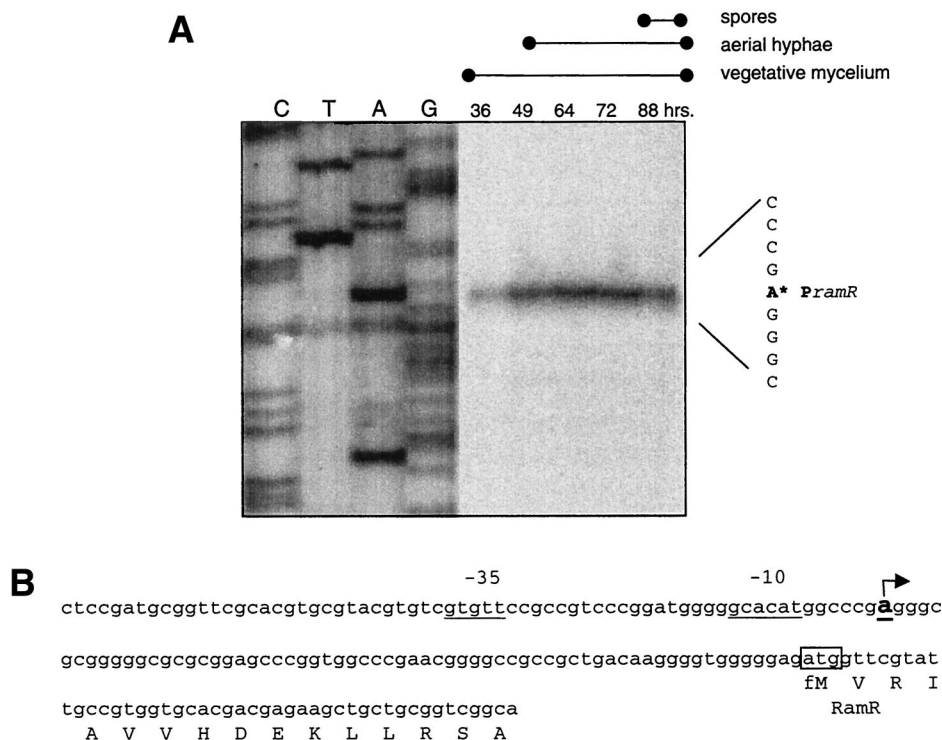


FIG. 2. Transcription analysis of *ramR*. (A) High-resolution nuclease S1 protection assay of the 5' end of *ramR* transcripts. RNA was isolated from wild-type *S. lividans* harvested after 36, 49, 64, 72, or 88 h of growth on solid R2YE medium. Developmental stages are indicated above the panel. The transcription of *ramR* was analyzed with a labeled probe (*ppr1*) directed at *ramR* and upstream regions (Fig. 1). The lanes C, T, A, and G represent a dideoxide sequence ladder of the same region generated with primer S1RF. The transcription start point is indicated by the asterisk. (B) Nucleotide sequence of the *ramR* promoter region indicating the *ramR* transcription start point and the start of the *ramR* coding sequence.

low levels of Red production in M512, observable only in older surface-grown cultures (>8 days), suggesting the presence of a weak promoter directly upstream of *ramA* (P3). To estimate the relative strength of promoter regions P1, P2, and P3, Red production of the various transformants was quantified during growth on solid media. Undecylprodigiosin was extracted from mycelium harvested during growth and quantified by determination of the  $A_{533}$ . The values are shown in Table 2. The relative level of Red production induced by P2 was very small compared to that induced by P1, while P3 induced an extremely low level of Red production that was detectable only after 67 h. On rich agar medium (R2YE), promoter activity in regions P1, P2, and P3 was detected with glucose, mannitol, or fructose as the carbon source (1% [wt/vol]). On the contrary, on solid minimal medium with either glucose or mannitol as the carbon source, the *ramCSAB* promoter-probe constructs did not elicit any Red production. Under these conditions, the abundance of Red production was observed with the control strain harboring pIJ2587 containing the constitutive  $P_{emE}$  promoter (data not shown).

In summary, promoter probing shows promoter activity in at least three regions of the *ramCSAB* operon: one upstream of *ramC* (P1), one in a region between -700 and -500 relative to *ramS* (P2), and one located directly upstream of *ramA* (P3).

The same promoter-probe constructs were introduced into *S. lividans* 1326. Although this strain produces low levels of undecylprodigiosin, pIJ2587 can be used for the probing of stronger promoters. P1 (pPst or pPstΔBK) elicited a high level

of Red production in *S. lividans* 1326. The level of Red production in *S. lividans* transformed with promoter-probe constructs harboring P2 (pPS2, pPS3, or pPS4) was increased in comparison with that in *S. lividans* carrying the empty vector pIJ2587 (data not shown). These results suggest a regulation that is highly similar to that in *S. coelicolor*.

**Temporal regulation of *ramCSAB* transcription.** To identify the transcription start in the region upstream of *ramC*, nuclease S1 mapping was performed with probe *pcp1* and RNA derived from *S. lividans* harvested during growth on R2YE

TABLE 2. Contribution of P1, P2, and P3 promoters to transcription of the *ramCSAB* operon

Time (h)	Red production <sup>a</sup>					Negative (M512)
	P1 (pPStΔBK)	P2			P3 (pPA1)	
		pPS4	pPS3	pPS2		
24	2	0	0	0	0	0
43	47	2	1	2	0	0
53	100	9	8	7	0	0
67	116	8	7	7	2	0

<sup>a</sup> To estimate the contributions of the various promoters (P1, P2, and P3) to transcription of the *ramCSAB* operon, Red production elicited by the promoter-probe constructs in host strain *S. coelicolor* M512 was determined quantitatively. Undecylprodigiosin was extracted from 100 mg of mycelium, harvested from surface-grown cultures with methanol. The absorption of the methanol fractions was measured at 533 nm. For comparison, a numerical value was calculated as (optical density at 533 nm per mg of cells) × 1,000.

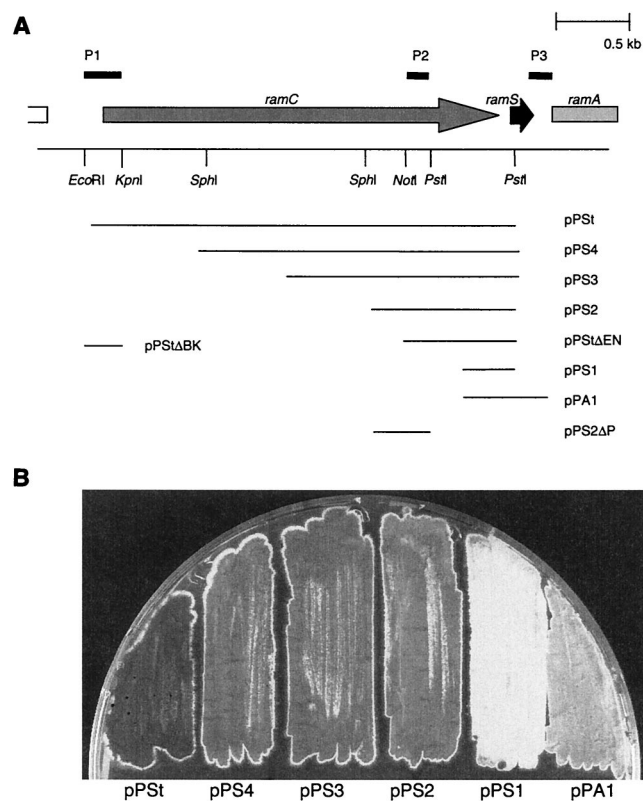


FIG. 3. Promoter-probe analysis of the *ramCSAB* operon. (A) The various fragments that were amplified by PCR and cloned in front of the promoterless undecylprodigiosin activator gene *redD* in vector pIJ2587 are indicated by the horizontal lines. These constructs allowed promoter activity to be monitored visually by the red pigment production elicited in host strain *S. coelicolor* M512 (M145 $\Delta$ *redD act11 orfIV*). The lines marked P1, P2, and P3 indicate the regions that showed promoter activity. (B) Bottom view of an R2YE plate with *S. coelicolor* M512 transformants carrying promoter-probe vectors pPS1, pPS4, pPS3, pPS2, pPS1, and pPA1 after 6 days of growth. pPS1 elicited a high level of Red production that became apparent after 30 h of growth. The level of Red production induced by pPS4, pPS3, and pPS2 was markedly lower and became apparent upon the onset of formation of aerial hyphae. pPS1 did not induce any Red production. The level of Red production induced by vector pPA1 was extremely low and was only visible after prolonged incubation. The Red production elicited by clones pPS4, pPS3, and pPS2 was similar to that elicited by deletion variants pPS2 $\Delta$ P and pPS1 $\Delta$ EN. The level of Red production of pPS2 $\Delta$ BK was identical to that induced by pPS1.

(Fig. 1). One strong band could be discerned, corresponding to a position 30 nucleotides (nt) upstream of the potential *ramC* translational start site (Fig. 4A). Consistent with promoter-probe data, transcripts were observed already during vegetative growth. Interestingly, transcript levels increased significantly upon the initiation of formation of aerial hyphae and decreased to a low level during aerial growth and spore production. Analysis of the potential *ramC* promoter regions did not reveal a clear  $-35$  and  $-10$  consensus sequence, but did identify a direct repeat (GTGACAGC-n26-GTGACAGC) potentially overlapping the *ramC* promoter (P1) (Fig. 4B).

To detect transcription into *ramS*, nuclease S1 protection assays were carried out with probe *pcp3* (Fig. 1). This probe overlaps the 3' end of *ramC* and the start of *ramS* and has a

64-bp nonhomologous tail at the 5' end, allowing discrimination between probe reannealing and full-length protection by mRNA. A strong band of approximately 300 nt was observed. The size of this band corresponds to full-length protection of the homologous part of the probe, confirming that the transcription of *ramS* is coupled to that of *ramC* (data not shown). Two additional bands with high intensity could be discerned (Fig. 5A), the first corresponding to a position 16 bp upstream of the *ramC* translational stop and the second corresponding to one of the potential *ramS* translational start sites (Fig. 5B). These results were also observed in experiments with different RNA isolates and confirmed by primer extension. Furthermore, such a high level of *ramS* transcripts was confirmed by Northern hybridization (data not shown). Since promoter-probe experiments could not detect any promoter activity in this area, we believe that these bands are due to processing of a larger transcript rather than de novo transcription.

Promoter-probe experiments revealed a weak internal promoter in the region between  $-700$  and  $-500$  relative to the *ramS* translational start site (Fig. 3). In an attempt to identify transcripts originating from this promoter region, nuclease S1 protection assays were performed with probe *pcp2* (Fig. 1). In addition to the band derived from full-length protection of the homologous part of the probe by the messenger, many relatively weak bands were detected (data not shown), most of which probably reflect processed *ramC* transcripts. This made it impossible to discriminate between de novo transcription from P2 and processed P1-derived transcripts. Since the same RNA was used for mapping of other genes, including *ramR* (Fig. 2A), which revealed no bands other than the full-length transcript, this is most likely due to specific turnover of *ramC* transcripts.

**Identification of a transcription terminator downstream of *ramS*.** Transcripts originating from P1 were readily detectable by S1 nuclease mapping with probe *pcp1*, *pcp2*, or *pcp3*. Conversely, by using probe *pcp4* to detect *ramA* transcripts, signals could only be detected after prolonged exposure of the phosphorimager screen. We took care to label the *ramC* and *ramA* probes (*pcp1* and *pcp4*, respectively) to very similar intensities. The relatively low level of *ramA* transcripts that were detected in *S. lividans* confirms our results published earlier (16). Sequence analysis revealed the presence of a large inverted repeat located directly downstream of the *ramS* ORF (Fig. 5B). The inverted repeat can potentially form a stable stem-loop structure in the nascent mRNA strand. This prompted us to map the 3' end of *ramCS* transcripts by nuclease S1 assays with probe *pcp5* (Fig. 1). A band of approximately 175 nt was detected (Fig. 5C), corresponding to a position at the 3' end of the inverted repeat. This indicates that transcription is terminated at this position and strongly suggests a role for the stem-loop structure as a termination signal (Rho independent).

**The effect of the *ramR* copy number on transcription of the *ram* operon.** The effect of an increased copy number of *ramR* on *ramCSAB* transcription was examined. P1 activity was analyzed in RNA derived from surface-grown cultures of *S. lividans* carrying *ramR* on the high-copy-number vector pUWL219 (pAF07) and the *S. lividans* *ramR::hyg* disruption mutant. The disruption of *ramR* caused a delay in the onset of formation of aerial hyphae of approximately 24 h compared to

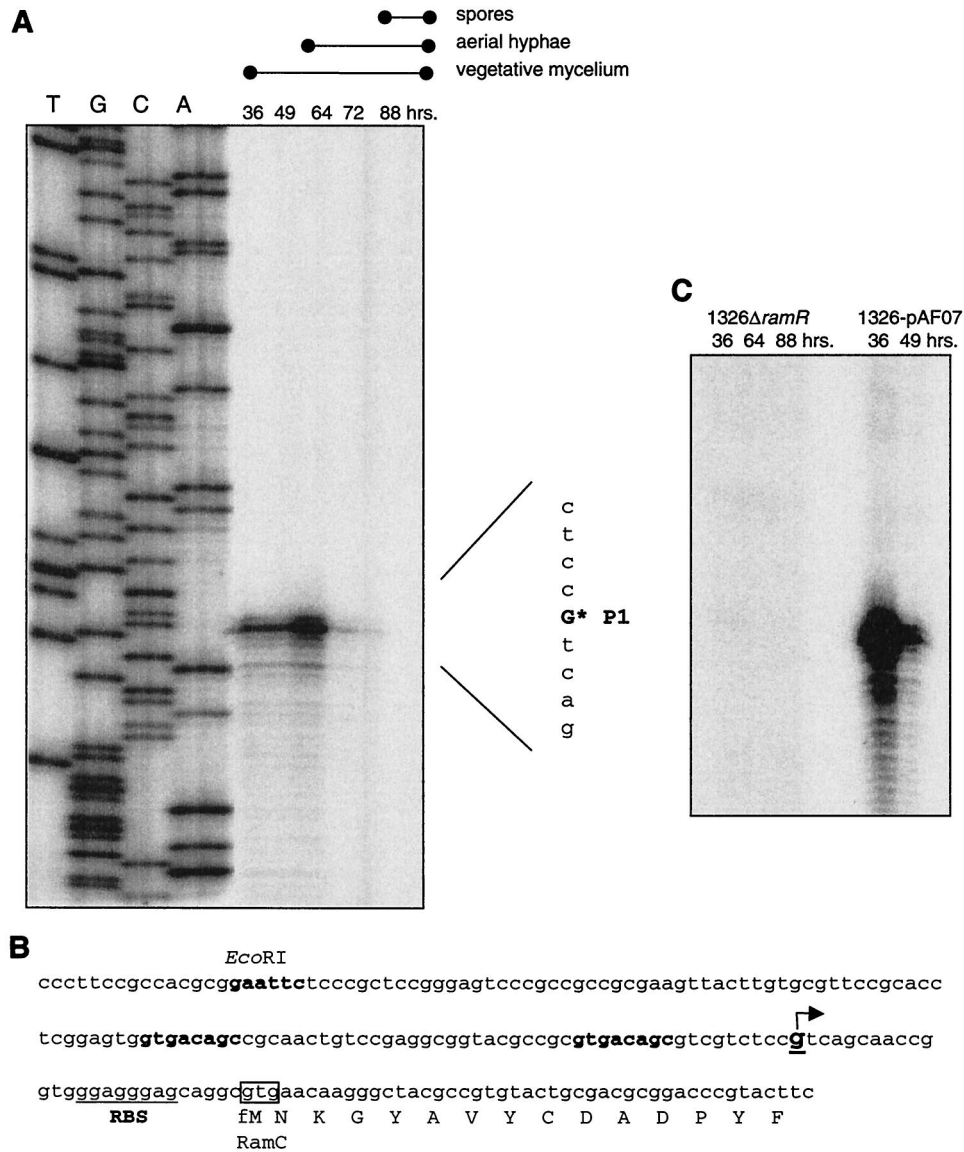
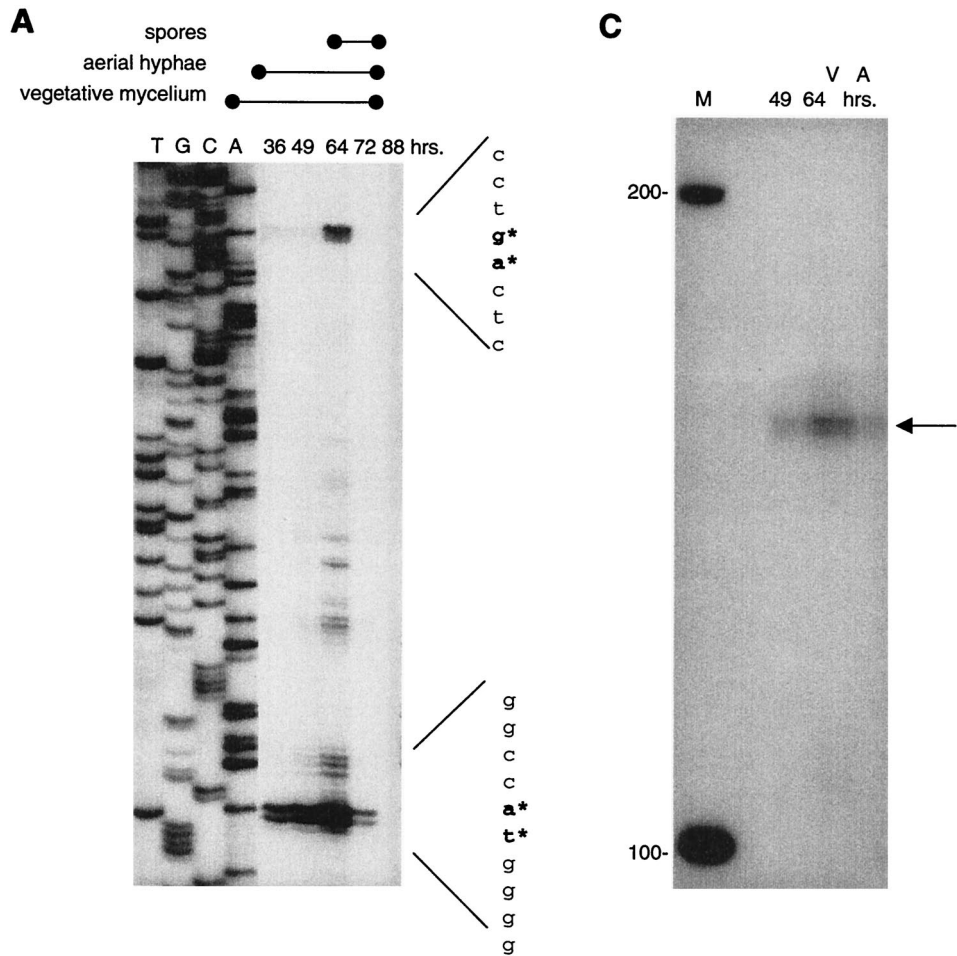


FIG. 4. Transcription analysis of the *ramCSAB* operon (A) The transcription start of *ramCSAB* transcription was analyzed by high-resolution S1 nuclease assay with probe *pcp1*. This probe encompassed the 5' end of *ramC* and upstream regions. The RNA was isolated at various time points (36, 49, 64, 72, and 88 h) during growth on solid R2YE. The developmental stages are indicated above the panel. The left half of the panel (lanes T, G, C, and A) shows the sequence ladder of the same DNA region that was produced with primer RCRV. The asterisk indicates the transcription start site. (B) Nucleotide sequence of *ramC* and upstream regions. The arrow indicates the transcription start site that was detected by nuclease S1 mapping. Shown in boldface is a direct repeat overlapping the putative promoter sequence. Underlined is the putative ribosome-binding site (RBS). (C) Transcription of *ramC* was assessed by nuclease S1 protection assays with *pcp1*. RNA derived from *S. lividans* carrying *ramR* on a high-copy-number vector (1326-pAF07) was isolated after 46 or 49 h of growth corresponding to formation of aerial hyphae. RNA derived from an *S. lividans ramR* deletion mutant (1326Δ*ramR*) was isolated from cells harvested after 36, 64, or 88 h of growth, during which aerial hyphae (64 h) and spores (88 h) were produced.

the wild type, *S. lividans* 1326. The aerial hyphae produced by this mutant strain subsequently formed mature and viable spores (16). The introduction of *ramR* on a high-copy-number plasmid (pAF07) into *S. lividans* resulted in an early onset of formation of aerial hyphae (Fig. 6). Analysis of P1 activity (*ramCSAB*) was performed by S1 nuclease protection assays with probe *pcp1*. The level of transcription from P1 was extremely high in RNA samples obtained from *S. lividans* carrying *ramR* on a high-copy-number vector (Fig. 4C). In accor-

dance with P1 activity in *S. lividans* 1326, transcript levels were found to decrease at later stages of formation of aerial hyphae. Conversely, the normally abundant P1-derived transcripts were not detected in the *ramR* disruption mutant, strongly suggesting that RamR is essential for *ramCSAB* transcription initiation.

**The *ram* genes are not expressed in *bld* mutants.** To relate *ramR* and *ramCSAB* transcription to a characteristic stage during development, transcription of the *ram* genes was analyzed



**B**

RamC  
gacgccccgccggactgcccgttcctgccgccgccgcccgaagcggcggccccaccctgactc  
D A P A G L P F L P P P R R S G G P L T R P H

RamS  
caggagccgtgagaaaagcacacccttgccgaacgaaaggaatgcgtc[atg]aacctgttcgacctgcag  
Q E P \* RBS fM N L F D L Q

NcoI  
tcgatggagacccccaaaggaagaggccatgggggacgtcgcgagaccggcagccgcgagcctcctgctc  
S M E T P K E E A M G D V E T G S R A S L L L  
RBS

tgccggcagacagcagcctgagcatcaccacctgtaactgacacaggggttgacggccacggcgacgtggt  
C G D S S L S I T T C N \*

gagtcatgtgccggcccgggcgccccgggttcggccggggcgccgacgcctgccggcgctcgacg

RamA  
tacggaggttctcgcc[atg]cgaaagagcaggccccgtgccc  
RBS fM R K S R P V P

in the *S. coelicolor* developmental mutants (*bldA*, *bldB*, *bldH*, *bldD*, and *whiG*) as well as in wild-type *S. coelicolor* strain M145. For this purpose, *S. coelicolor*, the *bld* strains, and *whiG* were grown on PCTE membrane-overlaid R2YE medium and harvested after 40 and 64 h, when *S. coelicolor* and *whiG* produced aerial hyphae. RNA was isolated and analyzed by nuclease S1 mapping, with *prp1* for the detection of *ramR* transcripts and *pcp1* for *ramCSAB* transcription. Although the *S. coelicolor ram* genes are highly similar to the *S. lividans ram* cluster, *S. coelicolor*-specific variants of probes *pcp1* and *prp1* were generated by performing the PCRs on the corresponding clones of the *S. coelicolor ram* cluster. In full analogy to *S. lividans*, transcription of *ramR* and *ramCSAB* in *S. coelicolor* M145 was readily detectable. Surprisingly, we were unable to detect any transcription from either Pr (*ramR*) or P1 (*ramCSAB*) in any of the *bld* mutants, while the transcription was detected in the earliest *whi* mutant, *whiG*. These findings show the intricate relationship between *ram* gene expression and initiation of formation of aerial hyphae.

## DISCUSSION

The initiation of morphological development is a poorly understood process that depends on a complex interplay of regulatory circuits, metabolism, cell signaling, and stress (6, 17). The *ram* gene cluster plays an important role in the initiation of differentiation of *S. lividans* and *S. coelicolor*. This cluster consists of genes encoding a sensor kinase (RamC), a small protein (RamS), the components of an ABC transporter (RamAB), and a response regulator (RamR) (16, 22). Homologous gene clusters have been identified in *S. griseus* (*amf*) and *Streptomyces avermitilis* (36, 37). Involvement of the *ram* genes in development has been demonstrated by both the accelerated development observed after this gene cluster was introduced as a single extra copy or on a multicopy plasmid into *S. lividans* and the negative effects on differentiation when disrupted. A functional correlation between the *ram* gene products is indicated by the similarity of the gene organizations of the *S. griseus amf* cluster and *S. lividans ram*, although the level of DNA sequence identity is relatively low. Transcription analysis showed that *ramC*, *ramS*, *ramA*, and *ramB* form one operon. By promoter probing, three promoter regions involved in transcription of this operon were identified. A strong promoter (P1) was identified directly upstream of *ramC*. Two promoters that have a very limited contribution to transcription of this operon were identified in a DNA segment situated

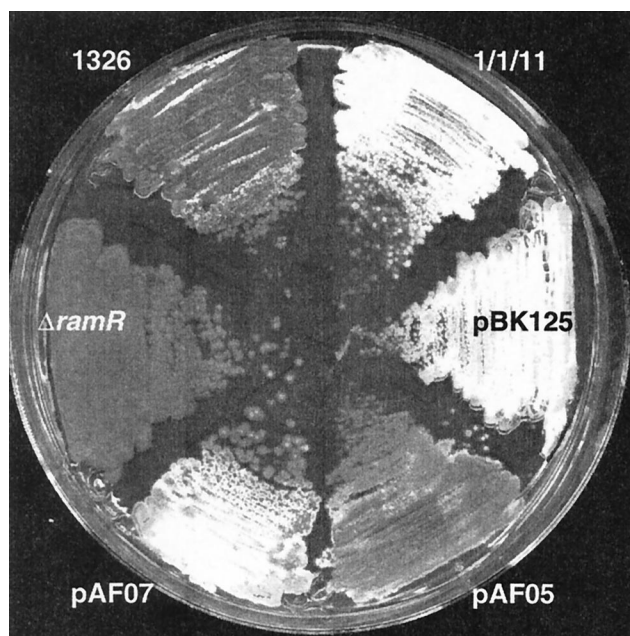


FIG. 6. Morphological effects induced by *ram* gene copy number. While *S. lividans* (strain 1326) initiated production of aerial hyphae after 3 days of growth on R2YE plates, abundant aerial hyphae were formed by *S. lividans* carrying the full *ram* cluster on the low-copy-number plasmid pIJ698 (1/1/11) or integrated as an extra copy in the genome at the  $\Phi$ C31 integration site (pBK125). In addition, *S. lividans* transformants carrying *ramR* in the high-copy vector pUWL219 (pAF07) also displayed an accelerated onset of development. The development of strains carrying *ramR* as an extra copy integrated at the  $\Phi$ C31 integration site in the genome (pAF05) was not accelerated. The development of *S. lividans* carrying an insertional mutation in *ramR* was delayed in comparison with that of wild-type strains.

between 500 and 700 bp upstream of *ramS* (P2) and directly upstream of *ramA* (P3), respectively.

Key steps during morphological development on rich media have been found to be less important for development on minimal media. This was shown by the conditional phenotype of the *bld* mutants (3, 24). A conditional *bld* phenotype was also observed for *S. lividans ramABR* mutants, which produced abundant aerial hyphae and spores when grown on minimal agar medium containing mannitol as a carbon source (16). Apparently, under these conditions, an alternative developmental pathway is used that is independent of the *ram* or *bld* genes (16, 17, 28). This was supported by promoter-probing

FIG. 5. Transcript processing and termination (A) High-resolution nuclease S1 protection analysis of the upstream region of *ramS* with probe *pep3*. RNA samples were isolated from *S. lividans* cultures grown on solid R2YE and harvested at various stages during development (36, 49, 64, 72, and 88 h). The developmental stages (vegetative growth, aerial mycelium, and spore production) are indicated above the panel. Lanes T, G, C, and A represent a sequence ladder generated with oligonucleotide RSRV, which was also used to generate the S1 probe. Since promoter-probe experiments did not indicate any promoter activity in this region, the transcripts detected have most likely been derived from processing of a larger transcript. (B) Nucleotide sequence and the amino acid sequences of the region between the 3' end of *ramC* and the 5' end of *ramA*. Underlined are the potential ribosome-binding sequences (RBS) upstream of the putative RamS and RamA translation initiation codons (indicated by the box). The horizontal arrows downstream of *ramS* mark an inverted repeat that may function as transcription terminator. Shown in boldface are the regions corresponding to bands detected by nuclease S1 mapping and primer extension analysis. (C) Mapping of the 3' end of *ramS* transcripts with RNA isolated from *S. lividans* grown on solid R2YE after 49 and 64 h. A band of approximately 175 nt in size was detected, indicating transcription termination corresponding to the position of an inverted repeat downstream of *ramS*.

data that showed that *ramR* and *ramCSAB* promoter activity occurs only on rich media and not on minimal medium containing either mannitol or glucose. On both types of media, sporulation was normal.

High-resolution nuclease S1 analysis of the upstream regions of *ramC* identified a single transcription start site 27 bp upstream of the RamC translational start (P1). Transcripts were detected during vegetative growth, but the transcript level was strongly upregulated upon the formation of aerial hyphae. In samples obtained during later stages of development of aerial hyphae and spores, transcript levels decreased to a low level. A clear  $-35$  and  $-10$  consensus sequence could not be identified upstream of the transcription start. Interestingly, a direct repeat (GTGACAGC-n26-GTGACAGC) was identified that possibly overlaps the putative  $-35$  and  $-10$  sequence. While the importance of this repeat for transcription regulation is unclear, it may well constitute a transcription factor-binding site. Similar repeat sequences were identified on the *S. coelicolor* genome at approximately 180 bp upstream of a putative ECF sigma factor gene (CAC33073) and approximately 20 bp upstream of a gene encoding a signal peptidase (CAC36379) ([www.sanger.ac.uk/Projects/S\\_coelicolor/](http://www.sanger.ac.uk/Projects/S_coelicolor/)). However, whether this implies a regulatory mechanism that is similar to that of the *ramCSAB* operon remains to be established.

A relatively large number of transcripts originating from P1 are terminated downstream of *ramS*, explaining the low level of transcription that has been detected for *ramA* (16). Transcripts terminate at a position corresponding to the 3' end of a large inverted repeat downstream of *ramS*. This region may form a stable stem-loop structure in the nascent messenger. Similar Rho-independent transcription-terminator mechanisms have been shown to provide an extra level of regulation for cotranscribed genes (39, 42). In addition, a high level of short *ramS* transcripts was found to accumulate during growth. This was shown by S1 nuclease mapping and Northern hybridization with RNA derived from several independent isolations. The stem-loop structure may be an important *cis*-acting structure element providing stability of the *ramS* mRNA by inhibiting 3'-5' exonuclease activity (2, 12, 19). This mechanism allows the differential expression of the *ram* genes within the polycistronic operon and may be important for the function of the *ram* gene products.

The transcription of *ramR* was shown to originate from a single start site 66 nt upstream of the apparent translational start codon. Transcription occurs during all growth stages and reaches a maximum during formation of aerial hyphae. The *ramR* promoter does not fall into the class of  $\sigma^{70}$ -like promoters and is thus likely to be dependent on a different RNA polymerase sigma factor for transcription (33). In addition, *ramR* transcription was shown not to be dependent on the developmental sigma factor  $\sigma^{\text{WhiG}}$ , since normal transcript levels were detected in *whiG* mutants.

RamR has often been suggested to play a role in the regulation of *ramCSAB* (16, 22, 37). The dependence of the *ramCSAB* genes on *ramR* has been demonstrated by complementation experiments. While the introduction of the full *ram* cluster as a single extra copy into *S. lividans* resulted in an accelerated development, the introduction of the *ramCSAB* genes does not bring about this effect. On the other hand, the introduction of *ramR* results in an accelerated development

only when placed on a multicopy vector and not as a single copy integrated in the genome (16, 22). The disruption of *ramR* eliminated virtually all P1 promoter activity, while the introduction of *ramR* on a multicopy vector resulted in a strong increase in *ramCSAB* transcript levels. This strongly suggests that RamR is involved in *ramCSAB* transcription. RamR may stimulate *ramCSAB* transcription indirectly, perhaps by stimulating other developmental genes that resort to a higher *ramCSAB* transcript level. Alternatively, RamR may act directly as a positive regulator of the *ramCSAB* operon, which would explain the conserved genetic architecture in *S. lividans*, *S. coelicolor*, and *S. griseus*. Perhaps a low, undetectable level of *ramCSAB* transcription from P1 in *ramR* mutants is sufficient to initiate development, albeit with a delay. Alternatively, promoter activity of the P2 and/or P3 promoter allows *ramR* mutants to initiate development.

Like most response regulators, RamR most likely requires phosphorylation by a sensor kinase for activity (32, 40). The *ramCSAB* transcription profile showing a sudden increase upon the initiation of formation of aerial hyphae may reflect the triggering of RamR activity by phosphorylation. A question that remains is what factors are responsible for activation of RamR. Unlike 62 of the total of 87 response regulator genes identified on the *S. coelicolor* genome ([www.sanger.ac.uk/Projects/S\\_coelicolor/](http://www.sanger.ac.uk/Projects/S_coelicolor/)), *ramR* is not localized adjacent to the cognate sensor kinase genes. There are examples of response regulator genes that do not lie adjacent to a histidine kinase gene that are also not activated by phosphorylation of the conserved aspartate residue (1, 13, 25). However, the integrity of the phosphorylation pocket and the conserved aspartate residue was shown to be crucial for AmfR activity in *S. griseus* and suggests that RamR also relies on phosphorylation for activation (36). Perhaps RamR is activated by one of the "lone" kinases encoded by genes that do not lie adjacent to an apparent response regulator gene. Alternatively, RamR may share a histidine kinase with another response regulator, as has been observed with the motility regulators CheB and CheY in *E. coli* and osmoregulators Ssk1p and Skn7p in *S. cerevisiae* (14, 21). An interesting candidate is a histidine kinase located on *S. coelicolor* cosmid 9E12, which is cotranslated with a *ramR* homologue and lies adjacent to orthologues of genes that in *S. griseus* are cotranscribed with *amfR*. The similarities in gene architecture between the *S. griseus* *amf* cluster, the *ram* cluster, and the genes downstream of the *S. coelicolor* *ramR* homologue may indicate a functional relationship, although this needs to be examined.

The transcription of *ramR* and the *ramCSAB* operon was studied in developmental mutants to relate the expression of these genes to a characteristic developmental stage. To our surprise, we were unable to detect any *ramR* or *ramCSAB* transcription in *bldD*, *bldA*, *bldB*, or *bldH* mutants. Conversely, transcription of *ramR* and *ramCSAB* was readily detected in samples obtained from the earliest *whi* mutant, *whiG*. The *bldD* gene product is known to repress the transcription of key developmental genes, such as *whiG* and *bldN*, (7). Mutation of the *bldD* gene leads to ectopic activation of sporulation genes, which could drastically disturb normal physiology and development. *ramR* and *ramCSAB* are the first developmental genes characterized as silent in *bldD*. Considering the absence of *ram* gene transcription in all of the *bld* mutants tested and its

presence in the *whiG* mutant, we suspect that *ram* transcription correlates temporally to the initiation of formation of aerial hyphae. The transcriptional coupling of *ramC*, *ramS*, and *ramAB* strongly suggests a functional relationship between the gene products. Recently, Ueda and coworkers demonstrated that the C-terminal part of AmfS partially restores the development of an *amfS* mutant when supplied exogenously. This led to the proposal of a model in which AmfA and -B are involved in the export and processing of AmfS, which functions as an extracellular signaling molecule (37). Additional experiments will be required to reveal the function of the *ram* gene products and the relationship to formation of aerial hyphae. The *ram* gene expression may be a final checkpoint prior to aerial growth and thus bridge the gap between the *bld* and *whi* mutants. Further insight into the function of the *ram* gene cluster will therefore improve our understanding of the mechanisms that regulate the switch from vegetative to aerial growth in the *Streptomyces* life cycle.

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