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## Function and control of the *ssg* genes in streptomyces

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# Chapter III

**Transcription of the sporulation gene *ssgA* is  
activated by the IclR-type regulator SsgR in a  
whi-independent manner in *Streptomyces*  
*coelicolor* A3(2)**

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## ABSTRACT

SsgA plays an important role in the control of sporulation-specific cell division and morphogenesis of streptomycetes, and *ssgA* null mutants have a rare conditionally non-sporulating phenotype. In this paper we show that transcription of *ssgA* and of the upstream-located *ssgR*, an *iclR*-type regulatory gene, is developmentally regulated in *S. coelicolor*, and activated towards the onset of sporulation. A constructed *ssgR* null mutant was phenotypically very similar to the *ssgA* mutant. The absence of *ssgA* transcription in this mutant is probably the sole cause of its sporulation deficiency, as wild-type levels of sporulation could be restored by the SsgR-independent expression of *ssgA* from the *ermE* promoter. Binding of a truncated version of SsgR to the *ssgA* promoter region showed that *ssgA* transcription is directly activated by SsgR; such a dependence of *ssgA* on SsgR in *S. coelicolor* is in clear contrast to the situation in *S. griseus*, where *ssgA* transcription is activated by A-factor, and its control by the SsgR orthologue, SsfR, is far less important. Our failure to complement the *ssgR* mutant with *S. griseus* *ssfR* suggests functional differences between the genes. These observations may explain some of the major differences in developmental control between the phylogenetically divergent species *S. coelicolor* and *S. griseus*, highlighted in a recent microreview (Chater and Horinouchi, 2003). Surprisingly, transcription of *ssgA* and *ssgR* is not dependent on the early *whi* genes (*whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*)

## INTRODUCTION

Streptomyces are soil-dwelling Gram-positive bacteria that have an unusually complex life cycle, which makes them particularly interesting for the study of bacterial development and evolution (Chater and Losick, 1997). During its life cycle, *Streptomyces* undergoes two apparently different events of cell division (reviewed in Flärdh and van Wezel 2003). Initially, cell division results in the formation of semi-permeable septa in the vegetative hyphae ('crosswalls') that delimit the multi-nucleoid hyphal cells. In solid-grown cultures, the reproductive phase is initiated by the formation of an aerial mycelium, with initially aseptate hyphae; in a later sporulation-programmed stage many septa are simultaneously formed, eventually resulting in mono-nucleoid spores (Chater, 2001). Recently, the genome sequences of *S. coelicolor* and *S. avermitilis* were elucidated, taking *Streptomyces* research into the genomics era (Bentley *et al.*, 2002; Ikeda *et al.*, 2003).

Genes involved in the transition from vegetative to aerial mycelium are called bald (*bld*) genes, characterised by the bald appearance of mutants due to their failure to produce aerial hyphae, and those involved in the subsequent processes leading to sporulation, are white (*whi*) genes, characterized by the white appearance of mutants due to a failure to complete sporulation. Six *whi* loci, designated *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*, identified by Chater (1972), are essential for the sporulation process (Flärdh *et al.*, 1999). The best characterised of these sporulation genes are *whiG*, encoding an RNA polymerase sigma factor (Chater, 1989); *whiB*, encoding a transcription factor with many homologues in streptomyces and mycobacteria (Soliveri *et al.*, 2000), and *whiH*, encoding a GntR-family transcription factor (Ryding *et al.*, 1998). The *whiH* mutant is phenotypically similar to a mutant in which the developmental *ftsZ* promoter had been inactivated (Flärdh *et al.*, 2000).

Recently, Chater and Horinouchi compared the developmental regulatory cascades in *Streptomyces coelicolor* and *Streptomyces griseus* (Chater and Horinouchi, 2003). These two organisms probably diverged from a common ancestor around 200 million years ago (Embley and Stackebrand, 1994), and an important difference between them is that *S. griseus* sporulates in submerged

culture. The signal for the onset of this still poorly understood process is the production of the  $\gamma$ -butyrolactone A-factor. In *S. griseus*, A-factor plays a more direct role in developmental control than the highly similar  $\gamma$ -butyrolactones (called SCBs) found in *S. coelicolor*: While A-factor non-producing mutants of *S. griseus* are defective in development and antibiotic production (Chater and Horinouchi, 2003; Horinouchi, 2002), these processes seem barely affected in  $\gamma$ -butyrolactone-deficient mutants of *S. coelicolor* (Takano *et al.*, 2001).

One of the key targets of A-factor in *S. griseus* is *ssgA* (Yamazaki *et al.*, 2003), a gene encoding a protein unique to sporulating actinomycetes that was originally identified as an effector of cell division in *S. griseus* (Kawamoto and Ensign, 1995b). SsgA plays an activating role in the production of sporulation septa, as its enhanced expression in *S. coelicolor* resulted in fragmentation of the mycelia in submerged cultures, producing spore-like compartments at high frequency (van Wezel *et al.*, 2000a). *ssgA* mutants of *S. coelicolor* and *S. griseus* are defective in sporulation, but form apparently normal vegetative septa (Jiang and Kendrick, 2000b; van Wezel *et al.*, 2000a). In total seven *ssgA*-like genes (*ssgA-G*) occur in *S. coelicolor*, and six in *S. avermitilis* (Flärdh and van Wezel, 2003). The *ssgB* gene was recently identified as a novel *whi* gene, and a null mutant produced large non-sporulating colonies (Keijser *et al.*, 2003).

Upstream of *ssgA* lies *ssgR*, a member of the family of *iclR*-type regulatory genes. This family includes IclR itself, the repressor for the isocitrate lyase gene (Sunnarborg *et al.*, 1990) and the acetate utilization operon (Galinier *et al.*, 1990) in *E. coli*, and the glycerol regulon repressor GyIR in *S. coelicolor* (Hindle and Smith, 1994). The IclR-type proteins, most often repressors, are characterised by an N-terminally situated helix-turn-helix (DNA binding) domain, and a C-terminal substrate binding domain, which is also important for oligomerization of the protein (Zhang *et al.*, 2002). None of the other *ssgA*-like genes is situated near an *iclR*-type regulatory gene. The SsgR homologues of *S. coelicolor*, *S. avermitilis*, and *S. griseus* (called SsfR) are highly similar, although the *S. griseus* homologue has a predicted N-terminal extension of 84 residues. Insertional inactivation of *ssfR* in *S. griseus* is phenotypically similar to the *ssgA* mutant, although *ssgA* transcription does not depend on SsfR *S. griseus* (Yamazaki *et al.*, 2003).

In this work, we study the transcriptional regulation of *ssgA* and *ssgR* in *S. coelicolor* A3(2) strain M145 and its early sporulation mutants *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*, together with the transcriptional dependence of *ssgA* on *ssgR*. We identified significant differences in the regulation of *ssgA* between *S. coelicolor* and *S. griseus*, and propose that this is one of the determinants of the morphological and developmental divergence between the two microorganisms.

## **MATERIALS AND METHODS**

### **Bacterial strains and culturing conditions**

The bacterial strains used in this work are listed in Table 1. *E. coli* K-12 strains JM109 (Sambrook *et al.*, 1989) and ET12567 (MacNeil *et al.*, 1992) were used for propagating plasmids, and were grown and transformed using standard procedures (Sambrook *et al.*, 1989). *Streptomyces coelicolor* A3(2) and its derivative M145, as well as the developmental (*whi*) mutants (Table 1), were obtained from the John Innes Centre strain collection, and *adpA* mutant M851 from E. Takano (Tübingen, Germany). M145 was used for transformation and propagation of *Streptomyces* plasmids. Preparation of media, protoplast preparation and transformation were performed according to Kieser *et al.* (2000). SFM medium was used to make spore suspensions. Minimal Medium (MM) agar plates containing 0.5% (w/v) mannitol, were used for RNA isolation; R2YE agar plates were used for regenerating protoplasts and, after addition of the appropriate antibiotic, for selecting recombinants. For standard cultivation of *Streptomyces*, and for plasmid isolation, YEME or TSBS (tryptone soy broth (Difco) containing 10% (w/v) sucrose), were used.

### **Plasmids and constructs**

The plasmids and constructs described in this paper are summarised in Table 2, and a map of the *ssgRA* gene cluster is shown in Figure 2A.

#### *1. General cloning vectors*

pIJ2925 (Janssen and Bibb, 1993) is a pUC19-derived plasmid used for routine subcloning. For cloning in *Streptomyces* we used the shuttle vectors pHJL401 (Larson and Hershberger, 1986), pWHM3 (Vara *et al.*, 1989) and pSET152

(Bierman *et al.*, 1992). All three vectors have the *E. coli* pUC19 origin of replication; maintenance in streptomycetes occurs via the SCP2\* *ori* (Lydiate *et al.*, 1985) (5 copies per chromosome) on pHJL401, the pIJ101 *ori* (50-100 copies per chromosome) on pWHM3, and the *attP* sequence (allowing integration at the attachment site of bacteriophage  $\phi$ C31) on pSET152. Plasmid DNA was isolated from ET12567 prior to transformation to *Streptomyces*. For selection of plasmids in *E. coli* ampicillin was used, except for pSET152 (apramycin); chloramphenicol was added for ET12567 transformants. For selection in *S. coelicolor* we used thiostrepton for pHJL401 and pWHM3, and apramycin for pSET152.

## 2. Construction of pGWR2 for in-frame deletion of *ssgR*

To create a construct for in-frame deletion of *S. coelicolor ssgR*, a 1400 bp *Bgl*II-*Bam*HI fragment containing *ssgR* and part of *ssgA* was inserted into pIJ2925 digested with *Bam*HI, and the approximately 280 bp *Nco*I-*Sph*I segment of *ssgR*

**Table 1.** Bacterial strains.

Bacterial strain	Genotype	Reference
<i>S. coelicolor</i> A3(2)	SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Kieser <i>et al.</i> , 2000)
<i>S. coelicolor</i> M145	SCP1 <sup>-</sup> , SCP2 <sup>-</sup>	(Kieser <i>et al.</i> , 2000)
M851	<i>adpA</i> ( <i>bldH</i> ) mutant of M145	(Takano <i>et al.</i> , 2003)
C72	<i>whiA71 pgl</i> <sup>+</sup> SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Chater, 1972)
C70	<i>whiB70 pgl</i> <sup>+</sup> SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Chater, 1972)
C71	<i>whiG71 pgl</i> <sup>+</sup> SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Chater, 1972)
C119	<i>whiH119 pgl</i> <sup>+</sup> SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Chater, 1972)
C17	<i>whiI17 pgl</i> <sup>+</sup> SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Chater, 1972)
C77	<i>whiJ77 pgl</i> <sup>+</sup> SCP1 <sup>+</sup> SCP2 <sup>+</sup>	(Ryding <i>et al.</i> , 1999)
GSA3	M145 $\Delta$ <i>ssgA</i> (::: <i>aadA</i> )	(van Wezel <i>et al.</i> , 2000a)
GSA4	M145 $\Delta$ <i>ssgA</i> + pGWS7	(van Wezel <i>et al.</i> , 2000a)
GSA5	M145 $\Delta$ <i>ssgA</i> + pGWR1	(van Wezel <i>et al.</i> , 2000a)
GSR1	M145 $\Delta$ <i>ssgR</i>	This paper
GSR2	M145 $\Delta$ <i>ssgR</i> + pGWR1	This paper
GSR3	M145 $\Delta$ <i>ssgR</i> + pGWS10	This paper
GSR4	M145 $\Delta$ <i>ssgR</i> + pGWS7	This paper
<i>E. coli</i> JM109	See reference	(Sambrook <i>et al.</i> , 1989)
<i>E. coli</i> ET12567	See reference	(MacNeil <i>et al.</i> , 1992)

(Figure 2A) was removed to create an in-frame deletion in the *ssgR* gene on the plasmid. For this purpose, the DNA was digested with *NcoI* and *SphI*, and the protruding ends were filled in (*NcoI*) or removed (*SphI*) using T4 DNA polymerase and dNTPs, followed by ligation and transformation. To ascertain we had created an in-frame deletion, the DNA sequence was determined and a 279 bp deletion was confirmed. Subsequently, the apramycin resistance cassette *aac(C)IV* (Kieser *et al.*, 2000) was inserted into the *EcoRI* site of the construct (outside the *ssgRA* insert), producing pGWR2. After transformation of the non-replicating construct to *S. coelicolor* M145, initial integrants (apramycin resistant) were selected, allowed to sporulate on SFM plates without antibiotics, and replicated non-selectively to allow a second recombination event to take place, and plated for single colonies. The latter were replicated to SFM containing apramycin, to screen for double recombinants, which should have lost the plasmid

**Table 2.** Plasmids and constructs.

Plasmid	Description	Reference
pHJL401	<i>Streptomyces/E. coli</i> shuttle vector (5-10 and around 100 copies per genome, respectively)	(Larson and Hershberger, 1986)
pSET152	<i>Streptomyces/E. coli</i> shuttle vector (integrative in <i>Streptomyces</i> , high copy number in <i>E. coli</i> )	(Bierman <i>et al.</i> , 1992)
pIJ2925	Derivative of pUC19 (high copy number) with <i>BglII</i> sites flanking its multiple cloning site	(Bibb <i>et al.</i> , 1985)
Q11	Cosmid clone containing the <i>ssgRA</i> gene cluster	(Bentley <i>et al.</i> , 2002)
pGWR1	pHJL401 + 1.3 kb <i>ssgR</i> fragment (-300/+1000, relative to the translational start site of <i>ssgR</i> )	This paper
pGWR2	Construct used for creating <i>ssgR</i> deletion mutant	This paper
pGWR3	pWHM3 + 1.3 kb <i>ssgR</i> fragment (-350/+950, relative to the translational start site of <i>ssgR</i> )	This paper
pGWR5	pHJL401 + 1.3 kb fragment harbouring <i>ssfR</i> of <i>S. griseus</i> (-300/+1000, relative to <i>ssfR</i> translational start)	This paper
pGWS6	pIJ2925 harbouring PCR product of oligonucleotides Q10 and Q11, inserted as a 237 bp <i>EcoRI-HindIII</i> fragment.	(van Wezel <i>et al.</i> , 2000a)
pGWS7	pSET152 harbouring <i>S. coelicolor ssgA</i> expressed from the <i>ermE</i> promoter	(van Wezel <i>et al.</i> , 2000a)
pGWS10	pSET152 harbouring 1.2 kb <i>ssgA</i> fragment (-625/+540 relative to the translational start site of <i>ssgA</i> )	This paper
pGWR11	pET15b-based construct for the expression and purification of full-sized SsgR-241	This paper
pGWR12	pET15b-based construct for the expression and purification of truncated SsgR-155	This paper



and hence have become sensitive to apramycin. About 30% of all apramycin sensitive colonies were sporulation mutants. A check of four sporulating and four non-sporulating double recombinants by PCR revealed that all sporulation mutants carried the expected 300 bp in-frame deletion, while sporulating colonies had a wild-type *ssgR* gene. One of the mutant colonies was selected, and designated GSR1. The location of the deletion is shown in Figure 2A.

### 3. Constructs for complementation experiments

For complementation of the *ssgR* mutant, plasmid pGWR1 was designed. This low-copy-number pHJL401-based vector harbours the PCR-generated -350/+950 region (relative to the *ssgR* translational start), including *ssgR* itself and approximately 300 bp promoter sequences; the oligonucleotides used for PCR were Q16 and Q11 (Table 3), designed such as to add *EcoRI* and *BamHI* restriction sites at the *ssgR* upstream and downstream end, respectively. A multi-copy derivative of pGWR1, designated pGWR3, contains the same insert in pWHM3. Plasmid pGWR5 is essentially the same construct as pGWR1, only with the corresponding region of *S. griseus ssfR* instead of *S. coelicolor ssgR*. Plasmids GWS7 and pGWS10 were used for expression of *ssgA*. pGWS7 (van Wezel *et al.*, 2000a) contains *ssgA* behind the constitutive *ermE* promoter in the integrative vector pSET152, while pGWS10 is a pHJL401 derivative, harbouring a 1.2 kb insert with *ssgA* preceded by its natural upstream (promoter) region. The insert of pGWS10 was generated by PCR with oligonucleotides Q18 and Q6 (Table 3), and corresponded to the -625/+540 section relative to the start of *ssgA*.

### 4. Constructs for the expression of His<sub>6</sub>-tagged SsgR

The SsgR expression plasmid pGWR11 was constructed by amplifying *ssgR* from the *S. coelicolor* genome with *Pfu* DNA polymerase and oligonucleotides Q2 and Q3 (Table 3), and cloned as an *NdeI-HindIII* fragment into pET15b, allowing the production of N-terminally His<sub>6</sub>-tagged SsgR; the full sized product was designated SsgR-241. For the expression of a truncated version of SsgR (called SsgR-155), containing the N-terminal 155 amino acids of the protein and lacking the TM signature, we made the pET15b-based expression construct pGWR12. The procedure was the same as for pGWR11, except that oligonucleotides Q2 and R155Bam were used (Table 3), the latter introducing a stop codon

immediately downstream of the CTC codon for Leu155. The PCR product was cloned as an *NdeI*-*BamHI* fragment into pET15b.

### **Expression and purification of N-terminally (His)<sub>6</sub>-tagged SsgR**

Plasmids pGWR11 and pGWR12 were used for the expression of full-sized SsgR (241 aa) and truncated SsgR (155 aa), respectively. For protein expression and purification, BL21 codonplus cells (Stratagene) transformed with either pGWR11 or pGWR12 were grown in LB broth at 30°C to an OD<sub>600</sub> of about 0.5, SsgR expression was induced by the addition of 0.1 mM IPTG, and the culture was incubated for a further 4-6 h. Cells were collected by centrifugation at 4°C, and pellets resuspended in resuspension buffer (50 mM Tris-HCl pH 7.5, 5 mM MgCl<sub>2</sub>, 300mM NaCl, and 10 mM imidazole). After sonication, the samples were spun down, and the supernatant and pellet analysed on a 10-12% SDS-PAGE gel. Gels were stained with Coomassie Brilliant blue in 7% ethanol/12% acetic acid.

Soluble SsgR-155 was purified using Ni-NTA affinity chromatography according to the Novagen protocol, and eluted with 200 mM imidazole. Samples were dialysed against standard buffer (50 mM Tris pH 7.5, 40 mM NH<sub>4</sub>Ac, 10 mM MgCl<sub>2</sub>, 1 mM DTT). Inclusion bodies containing SsgR-241 were harvested by centrifugation at 15,000 x g, solubilised and purified using the Pierce 6xHis B-Per kit, according to the manufacturer's instructions. After purification of SsgR-241 under denaturing conditions using Ni-NTA affinity chromatography, the purified protein was dialysed against standard buffer.

### **Mobility-shift assay**

Binding of SsgR to target DNA was studied in the following binding buffer: 20 mM Tris-HCl (pH 7.4), 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1mM EDTA, 1mM DTT, and 5% glycerol. To minimise nonspecific interactions, 50 ng of pBR322 and 100 µg/ml BSA were added. Reaction mixtures (20 µl) contained 3 nM [<sup>32</sup>P]-labelled DNA probe and 2 µl of diluted protein fraction (between 1-20 ng). After 10 min incubation at 30°C, 2 µl dye (50% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue) was added and samples were analysed on a non-denaturing 6% polyacrylamide gel in 1X TBE buffer. The DNA probe used was produced by PCR using oligonucleotides Q10 and [<sup>32</sup>P]-labelled Q11 (Table 3), and corresponded to the -195/+41 region relative to the *ssgA* translational start,

similar to *ssgA*-S1 (Fig. 1A). Electrophoresis was carried out at 4°C (4 h at 15 V/cm). After drying, gels were analysed in a phosphor-imager (BioRad).

### PCR conditions

PCRs were performed in a minicycler (MJ Research, Watertown, MA), using *Pfu* polymerase (Stratagene, La Jolla, LA), and the buffer provided by the supplier, in the presence of 5% (v/v) DMSO, with annealing temperature of 58°C. For oligonucleotides see next section and Table 3.

### Transcript mapping and probes

Mycelium was grown on MM or SFM agar plates with mannitol (0.5% w/v) as the carbon source, by plating spores onto presterilised cellophane discs. The RNA was purified from the surface-grown mycelium using the Kirby-based protocol (Kieser *et al.*, 2000), except that DNaseI treatment was used in addition to salt precipitation to fully eliminate DNA from the nucleic acid preparations. Phase-contrast light microscopy was used to assess the developmental stage of the

**Table 3.** Oligonucleotides.

Primer	DNA sequence	5' end	relative to
Q2	5' ct <b>gaattcatatg</b> gtgcagcgggcatgctgctgctc	+1	<i>ssgR</i>
Q3	5' ct <b>gaagctt</b> tctaactgatggcatcttgc	+781	<i>ssgR</i>
Q6	5' ct <b>gaagctt</b> caccgctgccttctgctggccgggctc	+540	<i>ssgA</i>
Q10	5' ct <b>gaattc</b> agcgggtgcagaacgagg	-195	<i>ssgA</i>
Q11	5' gtacggcatag <b>ggatccc</b> gggtctcgtag	+82	<i>ssgA</i>
Q16	5' ct <b>gaattc</b> gctcgtcgcgcagcactggtg	-338	<i>ssgR</i>
Q17	5' ctgggatcctactcgtgctcggcgacgcg	+47	<i>ssgR</i>
Q18	5' ct <b>gaattc</b> gacacatggcgcgctgg	-625	<i>ssgA</i>
R155Bam	5' gctag <b>ggatcct</b> cagaggggctgcaccgggtagcggctc	+465	<i>ssgR</i>
T7-AF	5' <u>tcggctaatac</u> gactcactatagggatgcggaaagagagctcctc	+41	<i>ssgA</i>
T7-Rrev	5' gcaagttcatgtcgcgagctcgt	-358	<i>ssgR</i>
T7-RF	5' <u>tcggctaatac</u> gactcactatacggagccgatcagagtcgcggac	+1	<i>ssgR</i>
<i>ssgA</i> -RT-for	5' atgagctttctcgtgtccgagg	+1	<i>ssgA</i>
<i>ssgA</i> -RT-rev	5' cagcggcaccagcttgtcggtg	+325	<i>ssgA</i>
<i>whiG</i> -RT-for	5' ggaacagctgatcctgcactac	+141	<i>whiG</i>
<i>whiG</i> -RT-rev	5' tcgctacgcgcgctccacggtg	+418	<i>whiG</i>
16S-RT-for	5' tcacggagagtttgatcctggctc	+20	<i>rrnA</i>
16S-RT-rev	5' cccgaaggccgtcatcctcacgc	+436	<i>rrnA</i>

Added T7 promoter sequences are underlined, restriction sites used for cloning presented in bold face. Restriction sites: **gaattc**, *EcoRI*; **aagctt**, *HindIII*; **ggatcc**, *BamHI*; **catatg**, *NdeI*. The *BamHI* site in Q11 occurs naturally in *ssgA*, other sites were designed. Location of 5' end of oligonucleotides (T7 promoter sequences not included) is relative to the start of the gene presented in the last column. The database accession number for *whiG* is SCO5621, for *rrnA* SCO4123.

surface-grown mycelium prior to harvesting and RNA isolation. Appropriate primers were labeled at their 5' ends with ( $\gamma$ - $^{32}\text{P}$ )-ATP by using T4 polynucleotide kinase before DNA probes were produced by PCR, after which high-resolution S1 nuclease mapping was carried out according to previously described protocol (Kieser *et al.*, 2000). Alternatively, ( $\alpha$ - $^{32}\text{P}$ )-UTP-radiolabelled RNA probes were produced with T7 RNA polymerase according to the Maxiscript kit (Ambion), and RNA protection assays were carried out using the RPAIII kit (Ambion). For each RNA protection assay, excess of probe was hybridised to 30  $\mu\text{g}$  of RNA. Protected fragments were analysed on denaturing 6% polyacrylamide gels, where desired alongside a DNA sequencing ladder, produced using the T7 sequencing kit (Amersham Pharmacia Biotech), with as sequencing primers the downstream primers used for generating the PCR-based *ssgA* and *ssgR* probes.

Probes used for transcript mapping were produced by PCR, using oligonucleotides described in Table 3; **(1) *ssgR-T7***, a T7 RNA polymerase-generated RNA probe with incorporated ( $\alpha$ - $^{32}\text{P}$ )-UTP, produced from a PCR fragment made using oligonucleotides T7-RF and T7-Rrev; the probe was designed against the -358/+1 region (relative to the *ssgR* translational start); **(2) *ssgR-S1***, a DNA probe encompassing the -338/+47 region (relative to the *ssgR* translational start), generated by PCR with oligonucleotides Q16 and  $^{32}\text{P}$ -labelled Q17; **(3) *ssgA-T7***, a T7 RNA polymerase-generated RNA probe with incorporated ( $\alpha$ - $^{32}\text{P}$ )-UTP, produced from a PCR fragment made using oligonucleotides T7-AF and Q10; the probe was designed against the -195/+41 region relative to the *ssgA* translational start; **(4) *ssgA-S1*** was generated by PCR on plasmid pGWS6, using  $^{32}\text{P}$ -labelled Q11 and the 17-mer universal ('forward') pUC primer, and corresponded to the -195/+82 region (relative to the *ssgA* translational start); the probe contains an approximately 50 nt non-homologous 3' extension to discriminate between full-length protection by RNA and experimental artefacts due to probe reannealing.

### RT PCR analyses

RT-PCR analyses were carried out using the SuperScript III one-step RT-PCR System (Invitrogen) for the analysis of RNA. RNA was isolated from mycelium grown on SFM agar plates with a cellophane overlay, on which all strains sporulated well (M145, M851) or a bit (*ssgR* mutant). The samples were

prepared after 24 h, 48 h, and 96 h, corresponding to vegetative growth, aerial growth, and sporulation, respectively. For each RT-PCR reaction 1 µg of RNA was used together with 0.5 µM (final concentration) of each primer. The program used was as follows: 30 min cDNA synthesis at 55°C, followed by 40 cycles of: 15" at 94°C (denaturing), 30" at 56°C (annealing) and 60" at 68°C (elongation). The reaction was completed by 5 min incubation at 68°C. Samples were tested on a 2% agarose gel in TAE buffer, and stained with ethidium bromide. The following combinations of oligonucleotides were used (Table 3): (a) *ssgA*-RT-for and *ssgA*-RT-rev for *ssgA*; (b) *whiG*-RT-for and *whiG*-RT-rev for *whiG*; (c) 16SRT-for and 16SRT-rev for 16S rRNA (to check the integrity of the RNA preparations). RT-PCR experiments without prior reverse transcription were performed on all RNA samples to assure exclusion of DNA contamination. Data were verified in several independent experiments.

### **Computer analysis**

Frame analysis was performed using the frameplot program (Ishikawa and Hotta, 1999), sequence alignments were performed using Clustal (Higgins *et al.*, 1996), Blast searches were performed at NCBI (<http://www.ncbi.nlm.nih.gov>), and transmembrane helix predictions using the TMPred program ([http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)).

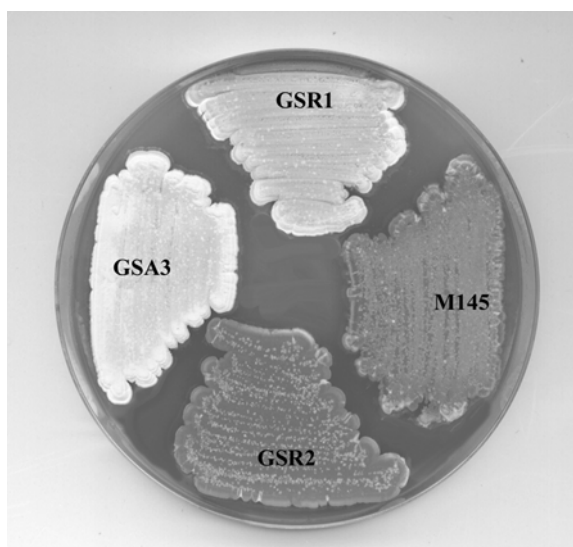
## **RESULTS**

### ***ssgR* is important for sporulation of *S. coelicolor***

The *ssgR* gene (*S. coelicolor* database reference SCO 3925) encodes a 241 amino acid IclR-type regulatory protein. Fourteen putative *iclR*-like genes could be identified within the *S. coelicolor* genome. An interesting feature of SsgR is its predicted transmembrane (TM) helix, YALGTVCAAIPITVGTTAATM (residues 185-205; high probability, as predicted by the TMPred server). Orientation is most likely N-terminus inside, which is consistent with the presence of a predicted helix-turn-helix (HTH) DNA binding domain in the N-terminal section of the protein (aa 19-40). Highly similar TM domains are also found in the SsgR homologues from *S. avermitilis* (SAV4268) and *S. griseus* (SsfR; AAF61237).

Another IclR-type regulator occurs in *S. coelicolor* with a highly similar putative TM domain (SCO2832, predicted TM sequence YAVGTVCAAVPITAGSAVGCL), which is the closest relative of SsgR in *S. coelicolor* (40% overall amino acid identity). However, its HTH domain is very different from that of SsgR.

To study the possible role of *ssgR* in *S. coelicolor* M145, an in-frame-deletion mutant of *S. coelicolor ssgR* was created, as described in the Materials and Methods section. This removed the approximately 280 bp *NcoI-SphI* fragment of *ssgR*, resulting in an in-frame deletion of the gene corresponding to aa 78-172. This mutant was designated GSR1. As shown in Figure 1, the mutant had a phenotype similar to that of the *ssgA* mutant GSA3; similarly, GSR1 formed aerial hyphae, but failed to produce spores on rich media such as R2YE or MM with glucose as the sole carbon source. Spores were produced on particular media, notably on MM with mannitol or on SFM, although at reduced levels and after prolonged incubation (Figure 1). Such an unusual conditionally white phenotype is also typical of the *ssgA* mutant (van Wezel *et al.*, 2000a).



**Figure 1. Phenotypes of the *ssgA* and *ssgR* mutants.** Sections of the relevant strains were streaked onto SFM plates, and photographed after 7 days. Strains used in this experiment: **M145**, Wild-type *S. coelicolor*; **GSA3**, *ssgA::aadA* disruption mutant; **GSR1**, *ssgR* in-frame deletion mutant; **GSR2**, GSR1 complemented by *ssgR* (pGWR1). Note the slightly grey appearance of the mutants, indicative of a low degree of sporulation on this particular medium.

### ***ssgR* is transcribed from a single developmentally regulated promoter**

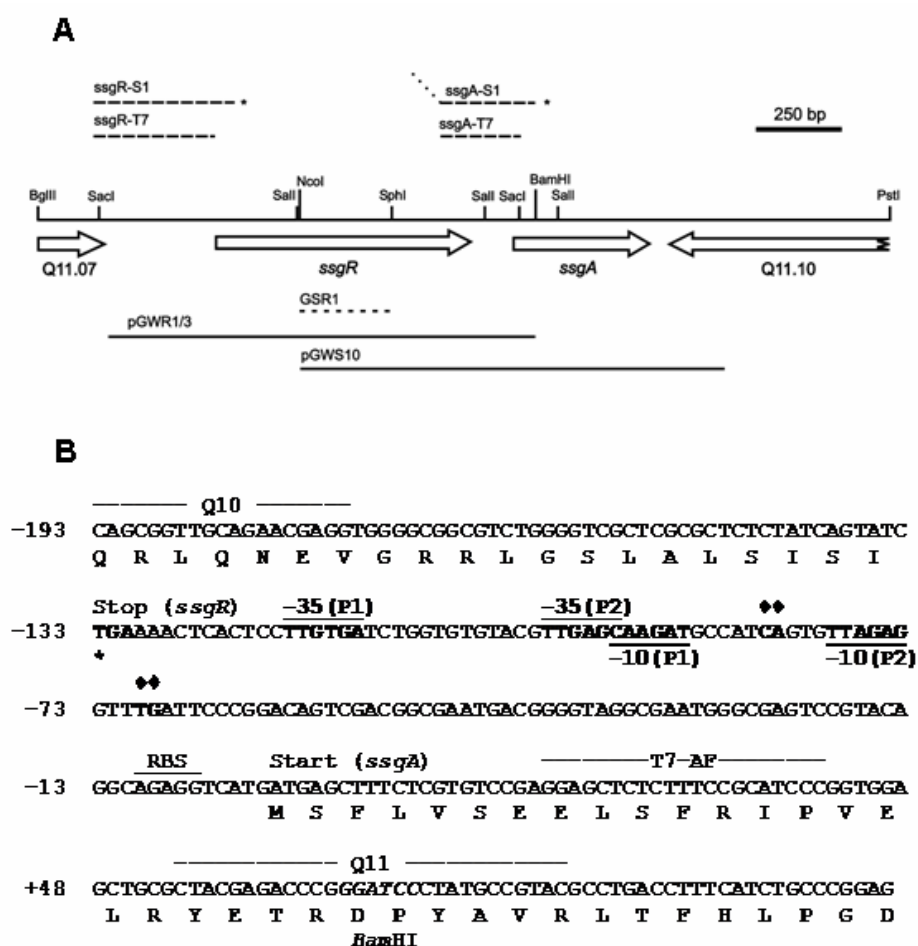
For transcriptional analysis of *ssgR*, and precise localization of the transcriptional start sites, transcript mapping experiments were performed on RNA isolated from *S. coelicolor* M145 grown on MM agar plates with mannitol as the sole carbon source. RNA was isolated at 12-24 hr intervals during 5 days, so as to provide representative samples to analyse transcription. The developmental stage of the samples was monitored using phase contrast microscopy.

Considering the low expression level of *ssgR* (see below) we used an end-to-end <sup>32</sup>P-labelled RNA probe for transcript mapping. RNA protection analysis using probe *ssgR*-T7 (-358/+1, relative to the *ssgR* translational start; Figure 2A) resulted in one major protected band, with a length of approximately 210 nt (Figure 3). Transcription of *ssgR* was upregulated after approximately 64 h, corresponding to the onset of sporulation (few spores were observed by phase contrast microscopy at this point). The experiment was repeated several times, also using RNA derived from cultures grown on SFM instead of MM agar plates. While the mycelium grew significantly faster on SFM than on MM, the developmental dependence of *ssgR* transcription was very similar in all experiments (not illustrated). Promoter probing experiments using the *redD* reporter system (van Wezel *et al.*, 2000c) confirmed the presence of promoter activity immediately upstream of *ssgR* (data not illustrated), ruling out the possibility that the transcripts had arisen from processing of an upstream-located promoter.

The exact transcriptional start point of the *ssgR* transcript was identified by comigration of the protected RNA probe together with a DNA sequencing ladder. The most likely transcriptional start site was identified as one of two A residues around 210 nt upstream of the translational start of *ssgR* (Figure 3B). This transcriptional start site is preceded by the sequence TAGAGT, which fully conforms to the consensus -10 sequence (TAGAPuT) for promoters recognised by the major RNA polymerase  $\sigma$  factor ( $\sigma^{\text{hrdB}}$ ) of *Streptomyces* (Strohl, 1992). However, we failed to identify a plausible -35 sequence.

Transcriptional analysis of the *S. griseus* *ssgR* orthologue, designated *ssfR*, showed that it is also transcribed from a single promoter, with a promoter sequence that is similar to that of *ssgRp*; the -10 sequences are almost identical (TAGAGT for *S. coelicolor*, TACAGT for *S. griseus*) and of the -50/-8 regions

(relative to the transcriptional start sites), 62% of the nucleotides are identical (S. Horinouchi, pers. comm.). Upstream of the *SsgR* homologue of *S. avermitilis* we identified a sequence that is highly similar to the *ssgR* promoter of

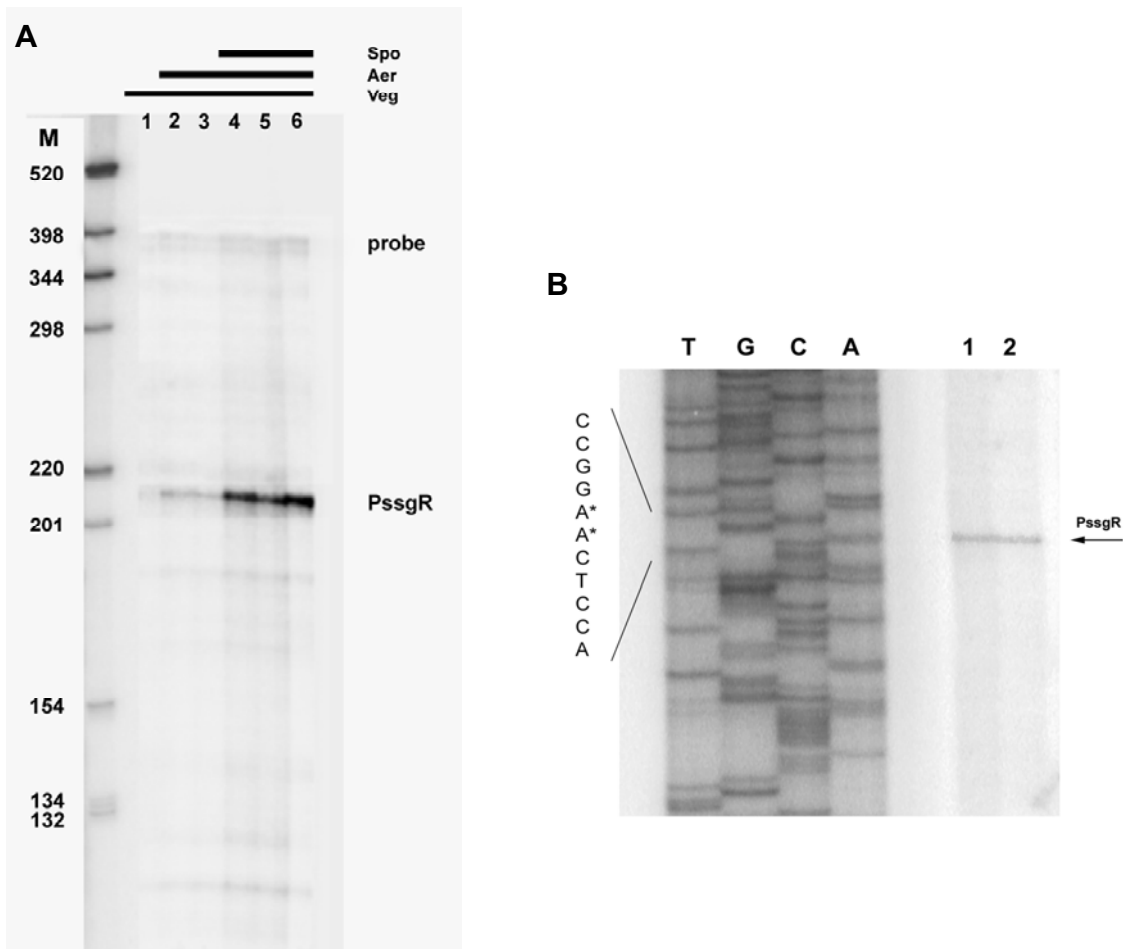


**Figure 2. Map of the *ssgRA* gene cluster, and sequence of the *ssgA* promoter region. 2A.** Map of *ssgRA*, and location of DNA fragments and probes. The *ssgRA* gene cluster is located on cosmid Q11. The most likely translational start of *ssgR* (ORF Q11.8; accession CAB46964) is a GTG triplet at nt position 4318648 on the genome, that of *ssgA* (Q11.9; accession CAB46963) an ATG triplet at nt position 4319504, resulting in proteins of 241 amino acids (SsgR) and 135 aa (SsgA), respectively. ORFs shown as arrows, indicating the direction of their transcription. Inserts of complementation constructs are shown by solid lines below the map. Dashed lines above the map represent the location of probes used for high resolution transcript mapping (See Materials and Methods, section 'transcript mapping and probes'). Extension (tilted dotted line) indicates 50 nt stretch containing non-homologous vector sequences. The approximately 280 nt deletion in the *ssgR* mutant GSR1 is represented by a dashed line below the sequence. **2B.** DNA sequence of the *ssgRA* intergenic region. Nucleotide numbering refers to the *ssgA* translational start site. ♦, transcriptional start sites (see Figure 4B); putative -35 and -10 sequences are overlined and underlined, respectively. Derived aa sequences corresponding to the end of *ssgR* and the start of *ssgA* are shown below the sequence. **RBS**, putative ribosome binding site. p1 and p2 refer to transcription start sites observed in surface-grown cultures. Important oligonucleotides are indicated (T7-AF and Q11 are complementary to the coding strand).



Transcription of the sporulation gene *ssgA* is activated by SsgR in *Streptomyces coelicolor* A3(2)

*S. coelicolor*, and this sequence therefore constitutes a likely *ssgR* promoter. This putative promoter (presumed -50/-1 region is 80% identical to that of *S. coelicolor ssgRp*) would start around nt position -240 relative to the start of *S. avermitilis ssgR*.



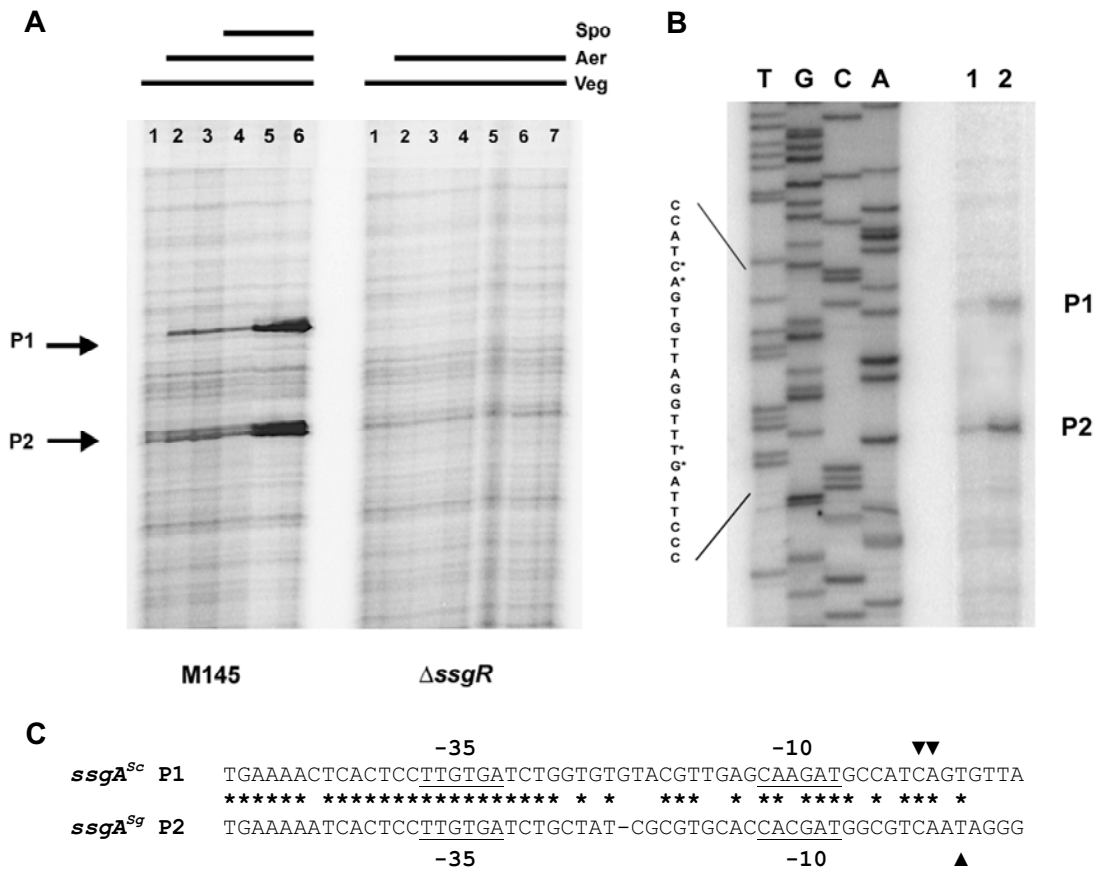
**Figure 3. Growth phase-dependent transcription of *S. coelicolor ssgR*.** **3A.** Transcript mapping experiment to identify *ssgR* transcripts. RNA was isolated from solid-grown MM + mannitol cultures of *S. coelicolor* M145, and analysed using high resolution transcription analysis. RNA time points: (1) 24 h (2) 36 h (3) 48 h (4) 64 h (5) 80 h (6) 96 h. Sample 2 corresponded to the onset of aerial mycelium formation, and sporulation started after approximately 64 h (sample 4). The RNA probe used for the RNA protection assays was *ssgR*-T7 (Figure 2A). While *ssgR* transcripts were already visible in samples 2 and 3, transcription of *ssgR* was strongly enhanced as soon as sporulation started. The location and developmental regulation of the *ssgR* promoter was verified with different probes and independent experiments (e.g. Figure 7B). **3B.** Determination of the exact transcription start site of *ssgR*. **TGCA**, *ssgR* nucleotide sequence ladder. Lanes 1 & 2 are the same samples as shown in lanes 2 & 3 in Figure 3A. Asterisks indicate the most likely transcriptional start site, coinciding with two A residues at nt positions -210 and -211, relative to the *ssgR* translational start site, respectively.

### Transcription of *ssgA* is dependent on *ssgR*

High-resolution mapping of *ssgA* transcripts was performed on the same RNA as was used for the analysis of *ssgR* transcription, with RNA probe *ssgA*-T7 (-195/+41, relative to the *ssgA* translational start; Figure 2AB). Two RNA-protected bands of approximately 125 nt and 110 nt were observed (p1 and p2, respectively; Figure 4A, left panel). The bands appear as double bands, with one nt difference. Such a duplication is often seen, *e.g.* for the *ftsZ* promoters (Flårdh *et al.*, 2000) and the *sigF* promoter (Kelemen *et al.*, 1996), probably as the result of an experimental artefact. While in all independent experiments it was difficult to detect *ssgR*-derived transcripts, *ssgA* transcripts could be readily detected.

*SsgA* transcription was induced after approximately 80 h, a time point where sporulation was well underway, while *ssgR* transcription was induced one time point earlier, corresponding to the onset of sporulation. Thus, transcription of *ssgR* is activated at least several hours prior to transcription of *ssgA*. We did not observe full-length protection of the probe in these experiments, which was confirmed by experiments using probe *ssgA*-S1 in Figure 7 (below), which carries a 50 nt non-homologous extension at its 3' end. Comigration of a DNA sequencing ladder showed that the transcriptional start sites corresponded to the approximate nt positions -84 and -69 relative to the translational start of *ssgA*, respectively (Figure 4B). The transcriptional start sites (full sequences in Figure 2B) are preceded by the sequences 5' TTGTGA-18 bp-CAAGAT-3' (for p1) and 5' -TTGAGC-15 bp-TTAGAG-3' (for p2), which show limited similarity to the consensus -35 and -10 sequences (5'-TTGACN-16-18 bp-TAGAPuT-3'; (Strohl, 1992) for promoters recognised by the major RNA polymerase holoenzyme of *Streptomyces*.

Since *ssgR* and *ssgA* mutants are phenotypically highly similar, a possible dependence of *ssgA* transcription on *ssgR* was investigated. For this purpose, transcriptional analysis was performed using RNA isolated from surface-grown MM mannitol cultures of GSR1, the congenic *ssgR* mutant of *S. coelicolor* M145. We repeatedly failed to detect significant levels of *ssgA* transcripts in these samples, suggesting that *ssgA* transcription is directly or indirectly dependent on *SsgR* in *S. coelicolor* (Figure 4A, right panel).



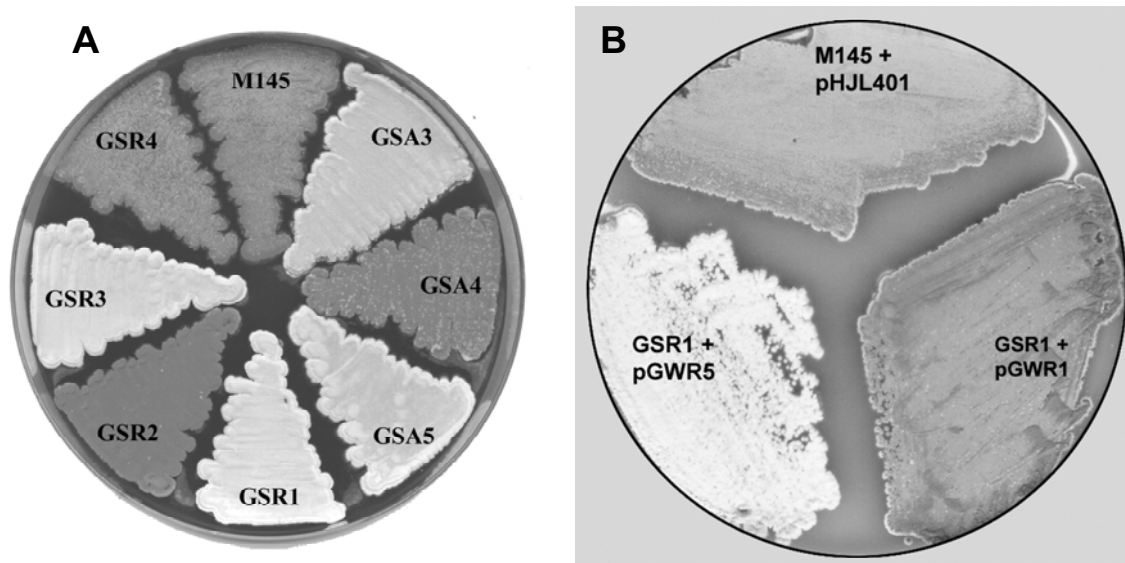
**Figure 4. Growth phase-dependent transcription of *S. coelicolor ssgA*, and dependence on *ssgR*.** **4A.** Transcript mapping experiment to identify *ssgA* transcripts in *S. coelicolor* M145 and its congenic *ssgR* mutant GSR1. RNA was isolated from solid-grown MM cultures. The RNA probe used for the RNA protection assays was *ssgA*-T7 (Figure 2A). Left panel: transcription in M145. The RNA used was the same as in Figure 3. Bands p1 and P2 correspond to *ssgA* transcripts. The intensity of both transcripts varied in a growth phase-dependent manner, with a maximum in lanes 5 and 6. Right panel: transcription in GSR1. RNA time points: (1) 24 h (2) 36 h (3) 48 h (4) 64 h (5) 80 h (6) 96 h (7) 120 h. Sample 3 corresponded to the onset of aerial mycelium formation. The relatively strong background is due to the use of a T7-generated RNA probe with incorporated nucleotides. The location and developmental regulation of the promoters was verified with several different probes and independent experiments (e.g. S1 mapping, Figure 7). **4B.** Determination of the transcription start sites of *ssgA*. **TGCA**, *ssgA* nucleotide sequence ladder. Lanes 1 & 2 correspond to the same samples as shown in lanes 2 & 3 in Figure 4A. Asterisks indicate the most likely transcriptional start sites for the two transcripts, coinciding with a A residue (for p1) and a G residue (for p2), at nt positions -84 and -69, relative to the *ssgA* translational start site, respectively. **4C.** Alignment of the *S. coelicolor ssgA* p1 and *S. griseus ssgA* p2 promoters. Putative -10 sequences underlined; arrowheads indicate the likely transcriptional start site. Asterisks indicate identical nucleotides between the two promoters. *S. griseus ssgA* p2 start site taken from (Yamazaki *et al.*, 2003).

The integrity of the RNA was confirmed by mapping the transcript of *ssgD* (SCO6722), one of the six *ssgA*-like genes in *S. coelicolor*, using the same RNA as in the experiments for mapping *ssgA* transcripts. The *ssgD* gene is expressed in all growth phases and in an *ssgR*-independent manner, from a single promoter (B. Traag & G.P. van Wezel, unpublished data). We observed no difference between M145 and GSR1 (data not illustrated). The absence of *ssgA*-derived transcripts in the *ssgR* mutant was confirmed by RT PCR experiments (see below).

### **Expression of SsgA restores sporulation to an *ssgR* mutant**

To further assess the dependence of *ssgA* transcription on *ssgR*, we analysed the morphological effect of *ssgR*-independent expression of *ssgA* in the *ssgR* mutant. For this purpose, two constructs were introduced into GSR1, one with *ssgA* preceded by its own (putatively SsgR-dependent) regulatory sequences, and one with *ssgA* positioned behind the SsgR-independent and constitutive *ermE* promoter. In a control experiment, we also introduced *ssgR* expression constructs in the *ssgA* mutant. In the latter case, no effect was expected. As additional controls, *ssgR* and *ssgA* mutants were complemented by wild-type copies of *ssgR* and *ssgA*, respectively. The results are shown in Figure 5A. As was anticipated, the *ssgA* and *ssgR* mutants could be complemented by the introduction of wild-type *ssgA* (on pGWS10, giving transformant GSA4) and *ssgR* (on pGWR1, transformant GSR2), respectively. This underlines that the non-sporulating phenotype of the *ssgR* and *ssgA* mutants is solely due to the respective gene deletions. Interestingly, morphological differentiation of the *ssgR* mutant could be fully restored by the expression of *ssgA* under the control of the SsgR-independent *ermE* promoter (GSR4 in Figure 5A). Therefore, the sporulation deficiency of the *ssgR* mutant is due to the lack of sufficient SsgA. In contrast, introduction of multiple copies of *ssgA* behind its own promoter did not complement the *ssgR* mutant (GSR3 in Figure 5A), providing additional evidence that the natural *ssgA* promoters are both inactive in an *ssgR* mutant background. Finally, as expected, introduction of pGWR1 in the *ssgA* mutant (transformant GSA5) had no apparent effect on development. As a control, all plasmids were transformed to the parental strain M145; these transformants showed normal sporulation, indicating that none of the plasmids had a negative effect on

sporulation. To test if *S. griseus* *ssfR* could also restore sporulation to the *ssgR* mutant, we introduced pGWR5 into GSR1. pGWR5 is essentially the same plasmid as pGWR1, except that it contains *ssfR* from *S. griseus* B2682 instead of *ssgR*. The non-sporulating phenotype of the resulting transformants shows that introduction of *ssfR* fails to complement the *S. coelicolor* *ssgR* mutant (Figure 5B).

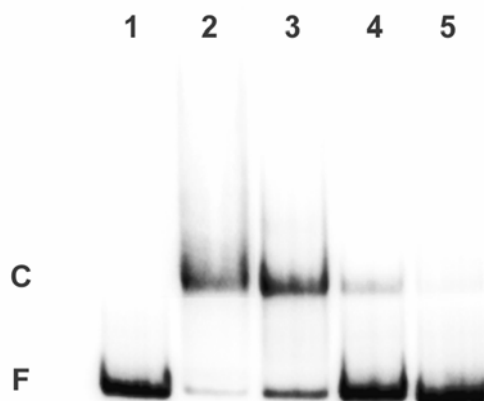


**Figure 5. Complementation of the *S. coelicolor* *ssgR* mutant.** **A.** Complementation of the sporulation-defective phenotypes of the *S. coelicolor* *ssgA* and *ssgR* mutants. Strains used in this experiment: **M145**, Wild-type *S. coelicolor*; **GSA3**, *ssgA* disruption mutant; **GSA4**, *ssgA* mutant complemented by pGWS7; **GSA5**, *ssgA* mutant harbouring pGWR1; **GSR1**, *ssgR* in-frame deletion mutant; **GSR2**, GSR1 complemented by *ssgR* (pGWR1); **GSR3**, GSR1 harbouring pGWS10 (*ssgA* with its own promoter); **GSR4**, GSR1 complemented by *ssgA* expressed from the SsgR-independent *ermE* promoter (pGWS7). Note that *ssgA* can only restore sporulation to the *ssgR* mutant if it is expressed from a promoter that is independent of intact SsgR. **B.** Complementation experiment using *S. griseus* *ssfR*. Transformants of GSR1 (the *ssgR* mutant of *S. coelicolor* M145) with pGWR5 (containing *S. griseus* *ssfR*) are shown, which fail to sporulate. Controls are full complementation of GSR1 by pGWR1, and wild-type level of sporulation by *S. coelicolor* M145 harbouring control plasmid pHJL401. For both experiments, strains were grown on SFM and incubated at 30°C for 7 days.

### **SsgR binds directly and specifically to the *ssgA* promoter region**

To analyse if SsgR could directly bind to the upstream region of *ssgA*, expression constructs were made to express and purify sufficient quantities of the protein for DNA binding assays (Materials and Methods). Construct pGWR11 was designed to produce full-length SsgR (241 aa, called SsgR-241) in *E. coli*, containing an N-terminal His<sub>6</sub>-tag for purification using Ni-NTA chromatography.

However, the protein was fully insoluble, and after purification of the protein from inclusion bodies using denaturing procedures, renaturation resulted in complete precipitation of the protein, with no detectable soluble protein (as judged by SDS-PAGE). The TMPred program identified a likely transmembrane (TM) region in SsgR, encompassing amino acids 185-205 (also present in the homologues of *S. avermitilis* and *S. griseus*). To remove the putative TM domain from the protein, we designed construct pGWR12 to express a shortened SsgR protein (155 aa, called SsgR-155). This resulted in soluble protein, which eluted from a Ni-NTA column in buffer containing 150 mM imidazole. The protein fraction containing pure SsgR-155 was tested for DNA binding activity in a mobility shift assay. Binding was observed to the DNA fragment SsgA-S1, which contains the -195/+45 section relative to the start of *ssgA* (Figure 2AB), producing a single and discrete DNA-protein complex (band C in Figure 6). Complete binding (lane 2) was observed with around 25 ng (approximately 1 pmol) of purified protein; at a 1:12 dilution (lane 4) virtually all DNA was in the unbound state, although some residual binding activity could be observed.



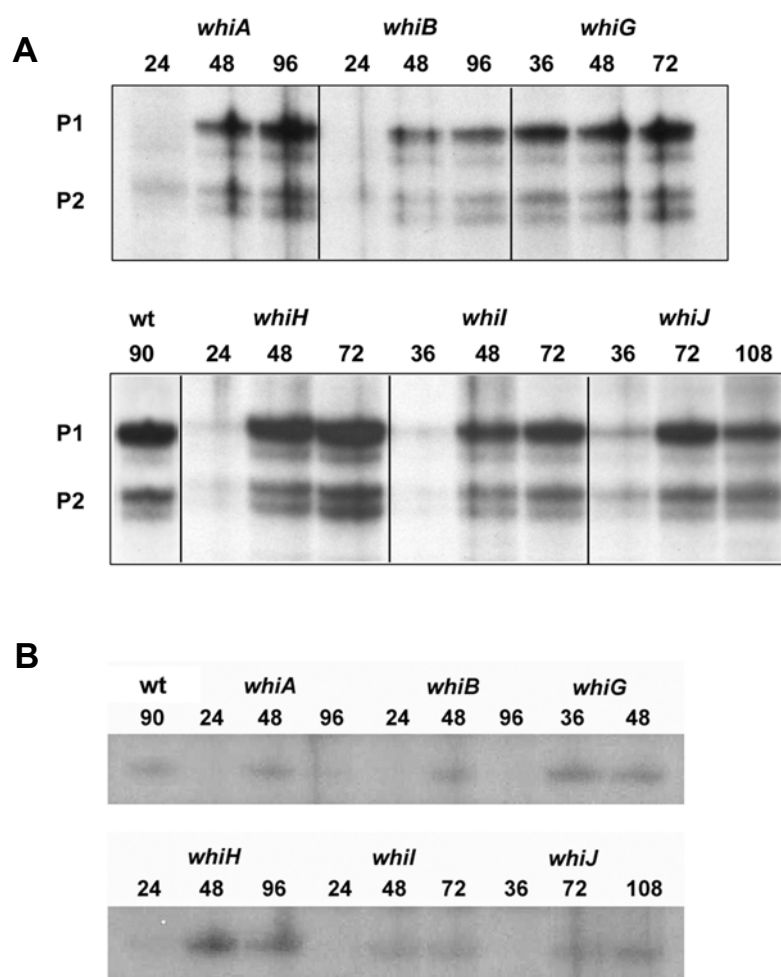
**Figure 6. Binding of SsgR to the *ssgA* promoter region**

Mobility shift assay showing binding of truncated His<sub>6</sub>-tagged SsgR-155 to the *ssgA* upstream region (-195/+41 relative to the start of *ssgA*). The DNA fragment used was <sup>32</sup>P-end-labelled *ssgA*-S1 (Figure 2A). Lanes: (1) control (no protein); (2) 25 ng SsgR-155; (3) 6 ng SsgR-155; (4) 2 ng SsgR-155; (5) same as lane 3, but with 5-fold excess of cold probe. **F**, free (unbound) fragment; **C**, complex of DNA and purified His<sub>6</sub>-tagged SsgR-155. Similar results were obtained when a smaller probe was used, which encompassed the -195/-75 region relative to the *ssgA* translational start site.

The mobility shift could be fully reversed by the addition of excess of cold probe (figure 6, lane 5), while the presence of a large excess of pBR322 did not affect binding. A fragment containing a shorter part of the *ssgA* promoter region (up to -75 relative to the start of *ssgA*) bound SsgR with similar affinity, narrowing down the SsgR binding site to the region between -195 and -75 relative to the start of *ssgA*. Specificity of SsgR for the *ssgA* promoter was underlined by its failure to bind to the SsgR promoter or to DNA fragments harbouring the start and middle part of the *ssgR* gene (not shown).

### **Transcription of *ssgA* and *ssgR* in sporulation (*whi*) mutants of *S. coelicolor***

Since *ssgA* controls the formation of sporulation septa, and expression of both *ssgA* and *ssgR* is induced during sporulation, a possible dependence of their transcription on the *S. coelicolor* sporulation genes *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ* was investigated. RNA from the corresponding *whi* mutants (Table 1) grown on MM agar plates with mannitol as the sole carbon source, was analysed by S1 nuclease mapping. Transcription of *hrdB*, which encodes the principal, essential  $\sigma$  factor of *S. coelicolor*, is expressed at a relatively constant level in *S. coelicolor*, and was monitored as an internal control (data shown in (Kelemen *et al.*, 1998)). S1 nuclease mapping of transcripts with DNA probe *ssgA*-S1 (-195/+82; Figure 2AB) revealed both *ssgA* transcripts in all *whi* mutants, showing that none of these genes is essential for *ssgA* transcription (Figure 7A). Expectedly, transcripts were absent in RNA isolated from vegetative mycelium. Developmental regulation of *ssgA* was also not significantly affected in any of the *whi* mutants, although *ssgA* transcription was slightly but reproducibly upregulated in the *whiH* mutant, which is developmentally stalled in a phase immediately prior to the onset of sporulation-specific cell division (Ryding *et al.*, 1998). Transcriptional analysis of *ssgR* in the *whi* mutants was done with DNA probe *ssgR*-S1, encompassing the -338/+47 region relative to the *ssgR* translational start site (Figure 2A). Similar to *ssgA*, *ssgR* appeared slightly upregulated in the *whiH* mutant, but was otherwise not significantly affected in any of the *whi* mutants (Figure 7B).



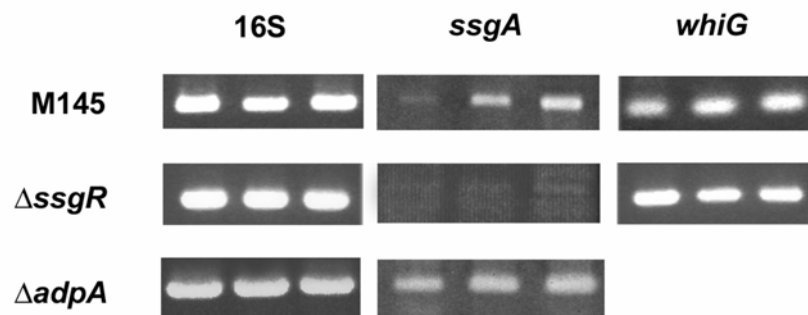
**Figure 7. Transcriptional analysis of *ssgA* and *ssgR* in *whi* mutants of *S. coelicolor*.** RNA was isolated from surface-grown MM/Mannitol cultures of the *S. coelicolor* sporulation mutants *whiA*, *whiB*, *whiG*, *whiH*, *whiI*, and *whiJ*, and analysed by nuclease S1 mapping. For each mutant, the first time point corresponds to the transition from vegetative to aerial growth, and the second and third time point to aerial growth. 7A. Transcript analysis of *ssgA*; 7B. Transcript analysis of *ssgR*. The probes used were *ssgR*-S1 and *ssgA*-S1, respectively (Figure 2A). As a control we used wild-type *S.coelicolor* A3(2), as this is the congenic parent of the *whi* mutants described in the paper. The results were essentially the same as those shown in Figures 3 and 4 (data not illustrated).

### RT-PCR analyses of developmental genes

In an independent set of experiments, we performed transcript analysis on RNA isolated from surface-grown MM Mannitol cultures by RT-PCR (Figure 8). To establish whether *ssgR* is important for earlier stages of aerial development, we analysed the transcription of the crucial regulatory gene *whiG*. The outcome of was very similar for M145 and the *ssgR* mutant, with approximately constant



transcript levels of *whiG* relative to the 16S rRNA in both strains, showing that *whiG* transcription was not significantly affected by the deletion of *ssgR* (Figure 8). Expectedly, there was a strong increase in *ssgA* transcript levels in RNA samples from surface-grown M145 towards the onset of sporulation (48 and 96 h), while we failed to detect *ssgA*-derived transcripts in the *ssgR* mutant. These results correspond well to the transcript mapping experiments presented in Figure 4A. Considering the dependence of *ssgA* on *adpA* (*bldH*) in *S. griseus*, we also analysed *ssgA* transcription in *adpA* mutant M851, which was characterised previously (Takano *et al.*, 2003). Interestingly, SsgA-derived transcripts were readily detected in this mutant; however, presence of *ssgA* transcripts in the 24 h sample (corresponding to vegetative growth) indicates that its regulation may be affected in the *adpA* mutant.



**Figure 8. Transcriptional analysis of developmental genes by RT PCR.**

Time points were (from left to right) 24h, 48h, and 96h, corresponding to vegetative growth, aerial growth and sporulation, respectively, for all three strains. Strains (vertical axis) were: *S. coelicolor* M145 and its *ssgR* and *adpA* mutant derivatives (GSR1 and M851, respectively). RNA was obtained from mycelium grown at 30°C on SFM plates (using a cellophane overlay). RNA (transcripts) analysed in the mutants are shown on horizontal axis (16S rRNA ("16S"), *ssgA*, and *whiG*). The RT-PCR experiments revealed normal *whiG* transcription in the *ssgR* mutant, and dependence of *ssgA* transcription on *ssgR*, but not on *adpA*. *whiG* transcription was not analysed in the *adpA* mutant. SFM agar plates were used to allow sporulation of the *ssgR* and *adpA* mutants.

## DISCUSSION

### ***ssgR* and *ssgA* are transcribed in a growth-phase dependent manner**

Our results show that *ssgA* and *ssgR* are sporulation genes, transcribed in a growth phase-dependent manner on solid-grown cultures. A constructed *ssgR*

mutant had a phenotype very similar to that of the *ssgA* mutant published previously (van Wezel *et al.*, 2000a), showing a conditional White phenotype (no sporulation except on mannitol-containing media). The *ssgR* gene was transcribed from a single growth-phase-dependent promoter (*ssgRp*) in solid-grown cultures of *S. coelicolor* M145. While steady-state transcript levels of *ssgR* were low under the conditions tested, the activation of *ssgRp* coincided with the onset of aerial mycelium formation, and transcript levels were strongly upregulated at the onset of sporulation. A very similar promoter was responsible for transcription of the *ssgR* orthologue in *S. griseus* (S. Horinouchi, pers. comm.). The sequence TAGAGT separated by 5-6 nt from the transcriptional start site, constitutes a -10 promoter consensus sequence that may be recognised by the principal sigma factor  $\sigma^{hrdB}$ , but a -35 consensus sequence for this probable promoter could not be identified.

*ssgA* is transcribed from two promoters (*ssgA* p1 and *ssgA* p2), separated by approximately 15 nt, suggesting overlap between them. The absence of read-through indicates that on solid-grown cultures, *ssgA* is transcribed only from these two promoters. Earlier experiments with liquid-grown cultures showed that *ssgA* is expressed at a low level and only after nutritional downshift, and co-regulated with *ssgR* from a full-length transcript (van Wezel *et al.*, 2000a). This experiment was repeated, again showing read-through from the *ssgR* promoter (data not illustrated). This discrepancy remains unexplained, but could reflect interesting differences between liquid- and solid-grown cultures. Both promoters were developmentally controlled, and maximal transcript levels were reached one time point later than those of *ssgR* transcripts, at a time corresponding to sporulation. Such a sporulation-specific expression conforms to a role in the activation of sporulation-specific cell division. Despite the similar developmental regulation and strength of *ssgA* p1 and p2, there is no obvious similarity between the respective promoter sequences.

Comparison to the *S. griseus* *ssgA* promoters revealed almost complete conservation between *ssgA*<sup>Sc</sup> p1 and *ssgA*<sup>Sg</sup> p2 (Figure 4C). This sequence is also highly conserved in *S. avermitilis*. However, *ssgA*<sup>Sc</sup> p2 shows no sequence similarity to *ssgA*<sup>Sg</sup> p1. Apparently, some elements of *ssgA* regulation are shared between *S. coelicolor* and *S. griseus*, while others are different (see the final paragraph).

### **Expression of *ssgA* is activated by SsgR**

Our failure to detect *ssgA* transcripts in the *ssgR* mutant indicated that the growth-phase-dependent induction of *ssgA* transcription is dependent on SsgR. This is in accordance with the observation that *ssgR* transcription is strongly induced one time point earlier than that of *ssgA*, at a time between aerial hyphae formation and the onset of sporulation; transcripts of *ssgA* itself were induced when sporulation was initiated. Interestingly, *ssgA* transcripts were more abundant than those of *ssgR*, as seen in multiple independent experiments. In further support of activation of *ssgA* transcription by SsgR, introduction of a plasmid expressing *ssgA* from the constitutive and SsgR-independent *ermE* promoter restored a wild-type phenotype to the *ssgR* mutant of *S. coelicolor*, while a similar plasmid harbouring *ssgA* with its natural promoter failed to complement the mutant. This suggests that while it is possible that other genes are regulated by *ssgR*, the sporulation deficiency of the *ssgR* mutant is solely due to lack of SsgA, and that *ssgA* transcription fully depends on SsgR. This is also supported by the observation that fragmentation of *S. coelicolor* in submerged culture, typical of transformants expressing *ssgA*, was induced by the introduction of pGWR3, a multi-copy plasmid harbouring only *ssgR* (data not illustrated). Interestingly, introduction of a plasmid harbouring *S. griseus* *ssfR* did not restore sporulation to the *ssgR* mutant, indicating significant functional differences between the respective gene products. SsgR of *S. coelicolor* belongs to the family of IclR-like transcriptional regulators (Zhang *et al.*, 2002). These proteins are characterised by an N-terminal DNA binding domain, and a C-terminal ligand binding domain, which is also important for oligomerization of the protein. Typically, binding of the substrate induces a conformational change, releasing the protein from its target sequence (Yamamoto and Ishihama, 2003; Zhang *et al.*, 2002). As an exception, the *Streptomyces* SsgR/SsfR proteins have a predicted transmembrane helix. Our experiments suggest that cleavage of this putative TM domain is required for its solubility, as only a truncated version of SsgR (155 amino acids long) was soluble. Whether SsgR also binds a substrate is unclear, but considering that on several occasions it was shown that the C-terminal section of IclR-type proteins is required for interaction between monomers, its removal is expected to affect the mode of binding of SsgR. The truncated SsgR-155 produced a single mobility shift on a DNA fragment

containing the *ssgA* promoter region, indicating that the SsgR dependence of the transcription of *ssgA* is mediated through direct *trans*-activation by SsgR. The binding site was narrowed down to the -195/-75 section relative to the *ssgA* gene. The region around the stop codon of *ssgR* constitutes a possible binding site, as it harbours a for streptomycetes unusually A/T-rich sequence (TGAAAACTCACTCC) that shows significant similarity to the consensus sequence TGAAAA(A/T)NNTTTPyPy for IclR-type binding sites (Pan *et al.*, 1996; Zhang *et al.*, 2002). Typically, IclR-type regulators bind to multiple IclR boxes, although we failed to observe additional binding by SsgR. We cannot rule out the possibility that this different behaviour is due to the absence of the C-terminal (interaction) part of the protein, which we had to remove to obtain soluble SsgR protein. We are currently analysing the mode of action and the activation of SsgR in more detail.

### **Interdependence of *ssgRA* and other developmental genes**

In *S. coelicolor* *whiA*, *whiB*, *whiG*, *whiI*, and *whiJ* mutants, the level and timing of *ssgA* and *ssgR* transcription are comparable to those found in the parental wild-type *S. coelicolor* A3(2) as well as those in *S. coelicolor* M145. Considering that *ssgRA* transcripts are found in all "early" *whi* mutants analysed, this strongly suggests that the gene cluster is not controlled by the classical *whi* genes. In a reverse experiment, we also showed that *whiG* is not significantly affected in an *ssgR* mutant, providing further support for the mutual independence of the *ssgRA* cluster on the one hand, and the early *whi* genes on the other. This independence is apparently supported by the observation that the white phenotype of the *ssgA* and *ssgR* mutants is medium-dependent, and that these genes as well as most of the *ssgA*-like genes are under carbon catabolite control, which is not the case for other *whi* genes (Chapter IV of this thesis). Further transcriptional analysis is required to assess whether the observed upregulation of *whiH* in the *ssgA* and *ssgR* mutants is significant. A possible explanation for the necessity of *whi* gene-independent expression of *ssgRA* is that the genes are also involved in the activation of sporulation-specific cell division under conditions where an aerial mycelium is not produced, in particular during submerged sporulation. Indeed, *ssgA* is essential for this process in *S. griseus*

(Kawamoto *et al.*, 1997), and its overexpression results in hyperseptation and a low level of submerged sporulation in *S. coelicolor* (van Wezel *et al.*, 2000a).

### **Differential regulation of *ssgRA* in *S. coelicolor* and *S. griseus***

How does the situation in *S. coelicolor* compare to that in *S. griseus*? In the latter organism, *ssgA* is dependent on AdpA, an A-factor-dependent transcriptional activator that is essential for development and streptomycin production (Ohnishi *et al.*, 2002). In contrast, *S. coelicolor scbA* mutants fail to produce the A-factor-like  $\gamma$ -butyrolactone SCB1, but show normal sporulation (Takano *et al.*, 2001), and *adpA* mutants sporulate normally on mannitol-containing media (Takano *et al.*, 2003). Since such sporulation is not possible in the absence of *ssgA*, its transcription is most probably not dependent on SCB1 or AdpA, which was confirmed by our observation that *ssgA* transcripts could be readily detected in an *S. coelicolor adpA* mutant. Rather, in *S. coelicolor* both *ssgA* promoters are directly dependent on activation by SsgR. While in *S. griseus* the activity of *ssgA*<sup>Sg</sup> p2 (virtually identical to *ssgA*<sup>Sc</sup> p1, Figure 4C) also depends on SsfR, that of *ssgA*<sup>Sg</sup> p1 does not, which results in significant expression of *ssgA* in an *ssfR* mutant (Yamazaki *et al.*, 2003). Therefore, regulation is clearly different in *S. griseus*, also illustrated by the significantly higher *ssgA* transcript levels in this organism, which is a prerequisite for submerged sporulation (van Wezel, unpublished data).

In summary, several important differences exist between the regulation of the *ssgA* orthologues in *S. coelicolor* (<sup>Sc</sup>) and that in *S. griseus* (<sup>Sg</sup>):

- (1) *ssgA*<sup>Sc</sup> fully depends on activation by SsgR, while activity of only one of the *ssgA*<sup>Sg</sup> promoters is reduced in an *ssfR* mutant.
- (2) The SsgR<sup>Sc</sup> and SsfR<sup>Sg</sup> proteins may be functionally different, as the gene from *S. griseus* fails to complement the *S. coelicolor ssgR* mutant.
- (3) It is unlikely that the A-factor-like molecule SCB1 plays a role in the regulation of *ssgA*<sup>Sc</sup>, while A-factor is essential for the regulation of *ssgA*<sup>Sg</sup> (through AdpA). This may at least partially explain the different impact of A-factor on the development of these organisms, because SCB1 (and on mannitol also AdpA) mutants of *S. coelicolor* sporulate normally, while A-factor is essential for sporulation of *S. griseus* (Takano *et al.*, 2001)).

(4) There is no detectable transcription of *ssgA*<sup>Sc</sup> in submerged culture under normal conditions, while it is strongly expressed in *S. griseus* (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b).

The expression level of *ssgA* has a major impact on mycelial morphology of both organisms (Kawamoto *et al.*, 1997; van Wezel *et al.*, 2000a; van Wezel *et al.*, 2000b), and the different expression levels of *ssgA* in these organisms thus provides a possible explanation for their strong morphological differences in submerged cultures, and this may be one of the main reasons why *S. griseus* is able to sporulate in submerged culture, but not *S. coelicolor*. We are currently investigating the exact roles of SsgA and SsgR in the sporulation process, with focus on their cellular localizations, protein structures and molecular modes of action.

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