Identification and characterization of developmental genes in streptomyces

Zhang, L.

VI

The SARP-family regulator AfsR controls colony size and streptomycin production in *S. griseus* IFO13350

Le Zhang, Erica G. Wilson, Changsheng Wu, Young Hae Choi, Hiroshi Otani, Yasuo Ohnishi and Gilles P. van Wezel

ABSTRACT

AfsR is a regulatory protein that has been implicated in the control of development and/or secondary metabolism in streptomycetes. The proposed model is that it may influence the production of the γ-butyrolactone A-factor, which acts as a signal for the onset of development and antibiotic production in *S. griseus*. The spontaneous mutant AFN of *Streptomyces griseus* fails to develop or produce streptomycin due to almost complete block of A-factor production. Sequencing of this mutant suggestively revealed a truncation of the *afsR* gene, by mutation of Trp codon 881 (TGG) to a TAG stop codon. An *afsR* mutant was created, which showed accelerated development when grown as single colonies. Introduction of additional copies of either *afsR* or truncated mutant *afsR** led to increased streptomycin production and much larger colonies. However, we failed to see a correlation between A-factor production level and the presence or absence of *afsR* or *afsR**. Since introduction of *afsR** into the *afsR* null mutant developed normally, we conclude that the *afsR** mutation was not the (only) cause of the AFN phenotype.

INTRODUCTION

Streptomycetes and other actinobacteria are a predominant source of natural products for agricultural, biotechnological and medical applications (Hopwood, 2007). The production of natural products is closely correlated with the developmental program, and the signals that control development therefore also affect the secondary metabolism (Liu *et al.*, 2013; van Wezel and McDowall, 2011). The mycelial streptomycetes produce a branching network of hyphae in their habitat, the soil. These vegetative or substrate hyphae are divided by crosswalls, and hence *Streptomyces* are a rare example of a multicellular bacterium (Claessen *et al.*, 2014). On nutrient depletion, streptomycetes initiate a complex developmental program, whereby the vegetative mycelium serves as a substrate to build a second mycelium consisting of so-called aerial hyphae. Eventually these aerial hyphae differentiate to produce chains of spores. Mutants that fail to develop aerial hyphae are called bald or *bld* mutants (reflecting the lack the fluffy white aerial mycelium), and mutants that fail to sporulate are called white or *whi* mutants, as they do not produce the grey spore pigment.

A-factor (2-isocapryloyl-3R-hydroxymethyl-gamma-butyrolactone) is a hormone-like autoregulatory molecule that acts as a trigger of differentiation and streptomycin production in *Streptomyces griseus* (Horinouchi and Beppu, 2007). A-factor binds to the A-factor receptor protein ArpA, thus relieving inhibition by ArpA of transcription of the global regulatory gene *adpA* (Ohnishi *et al.*, 1999; Ohnishi *et al.*, 2005). AdpA directly activates transcription of a whole range of genes involved in development and secondary metabolism (Ohnishi *et al.*, 1999; Ohnishi *et al.*, 2005). Upon reaching a threshold level, A-factor synchronizes the activities of individual cells within a mycelium and likely also nearby cells (Horinouchi, 2002). The enzyme that synthesizes A-factor is AfsA (Kato *et al.*, 2007). An intriguing A-factor related regulatory system is formed by the sensory kinase AfsK and its cognate regulator AfsR, first discovered as a system that controls development in response to glucose (Umeyama *et al.*, 1999). AfsK is a serine/threonine kinase, which phosphorylates AfsR as well as itself (Hempel *et al.*, 2012; Umeyama *et al.*, 1999). AfsR is a member of the so-called SARP regulators, to which also many pathway specific activators of antibiotic production belong (Wietzorrek and Bibb, 1997), but has additional domains ATPase activity and a phosphorylation receiver domain (Hong *et al.*, 1991). AfsR binds to the promoter of *afsS*, a small gene located immediately downstream of *afsR* (Lee *et al.*, 2002), thereby activating its transcription (Tanaka *et al.*, 2007). The precise function of AfsS is unclear,

but it shows similarity to σ factors (Lee *et al.*, 2002) and its enhanced expression stimulates antibiotic production in *S. coelicolor, S. lividans* and *S. griseus* (Atsushi *et al.*, 1994; Floriano and Bibb, 1996; Vögtil *et al.*, 1994).

Previous research identified a spontaneous A-factor non-producing mutant (AFN) of *S. griseus* NRRL B2682 (Biró *et al.*, 2000). Mutant AFN is unable to form aerial hyphae or to produce streptomycin, likely due to its very limited A-factor production, and sporulation could be restored to *S. griseus* AFN by supplementing cultures with A-factor (Birkó *et al.*, 2009). Interestingly, the transcription of the key A-factor synthesis gene *afsA* was significantly up-regulated in *S. griseus* AFN as compared to its parental strain, which suggests that the defect in production was downstream of *afsA* (Birkó *et al.*, 2007). We have now sequenced the genome of *S. griseus* AFN, which revealed a non-sense mutation in the *afsR* gene, whereby the codon for Trp881 was changed to a stop codon. In this study, we introduced this so-called *afsR** mutation into a newly created *afsR* null mutant of the model strain *S. griseus* IFO13350. This showed that the *afsR** mutation could not explain the AFN phenotype as it did not cause any defect in development or streptomycin production, and that A-factor production was not affected. However, increasing the *c*opy number of *afsR* and flanking region resulted in a large colony phenotype and enhanced streptomycin production.

MATERIALS AND METHODS

Bacterial strains and media

Bacterial strains used in this work are listed in Table 1. *Escherichia coli* strains JM109 (Sambrook *et al.*, 1989) and IR539 (Suzuki *et al.*, 2011) were used for routine cloning procedure and specific sites-methylated DNA isolation respectively. Transformations in *E.coli* were selected in L broth (LB) media containing proper antibiotics at 37°C (Sambrook *et al.*, 1989). *Streptomyces griseus* IFO13350 was used as parental strain to construct mutants described in this work. YEME (yeast extract - malt extract (Kieser *et al.*, 2000)) or TSBS (tryptone soy broth (Difco) containing 10% (w/v) sucrose) were used for standard cultivation. R5 agar plates (Kieser *et al.*, 2000) were used for regeneration of protoplast and with appropriate antibiotics for selection of recombinants. Spore suspensions, morphological characterization and all microscopy analysis were performed on R5 and YMPD (1.5% glucose) agar plates as described (Colson *et al.*, 2008; Umeyama *et al.*, 1999). Bennett maltose agar plates (Hirano *et al.*, 2008) were used to detect streptomycin production.

Table 1. Bacteria strains.

Bacteria strains	Genotype	Reference
E. coli JM109	See reference	(Sambrook *et al.*, 1989)
E. coli IR539	See reference	(Suzuki *et al.*, 2011)
S. griseus IFO13350	See reference	(Ohnishi *et al.*, 2008)
GAL74	IFO13350 Δ*afsR*	This work
GAL75	IFO13350 + pHJL401	This work
GAL76	IFO13350 + pGWS781	This work
GAL77	IFO13350 + pGWS782	This work
GAL78	GAL74 + pHJL401	This work
GAL79	GAL74 + pGWS781	This work
GAL80	GAL74 + pGWS782	This work

Plasmids and constructs

All plasmids and constructs are summarized in Table 2 and oligonucleotides in Table 3.

Table 2. Plasmids and Constructs

Plasmid and constructs	Description	Reference
pWHM3	*E. coli*/*Streptomyces* shuttle vector, multi-copy and very unstable in *Streptomyces*	(Vara *et al.*, 1989)
pHJL401	*E. coli*/*Streptomyces* shuttle vector, 5-10 copies in *Streptomyces* and around 100 copies in *E. coli*	(Larson and Hershberger, 1986)
pUWL-Cre	Plasmid expressing Cre-recombinase	(Fedoryshyn *et al.*, 2008)
pGWS753	pWHM3 containing flanking regions of *S. griseus* IFO13350 SGR_3012 with apraloxP-*Xba*I inserted between them in pWHM3 EcoRI-HindIII	This work
pGWS781	pHJL401 with 3.9kb fragment harboring *afsR* and *orf4* (SGR_3012) behind native promoter of *afsR*	This work
pGWS782	pHJL401 with 3.1kb fragment harboring truncated *afsR* (*afsR**) and its native promoter region	This work

Table 3. Oligonucleotides.

Name	**5'-3' sequence**[#]
afsR_LF-1383	GTCA**GAATTC**GAGCACGTTGCCGACGCCGATC
afsR_LR+6	GTCAGAAGTTATCCATCACC**TCTAGA**GTCCATTGATGCCCCCTGTCCTG
afsR_RF+2920	GTCAGAAGTTATCGCGCATC**TCTAGA**GGCTGACCGCCGCGGAGGCGTTC
afsR_RR+4367	GTCA**AAGCTT**CTGATGCGGGGGGCCACGATG
afsR_F-153	AGACAAGGCAGACTGTTGAG
afsR_R+2964	AGTGCGCCGATAAACGATTG
afsR_F-480	CATGGAATTCGAACTTGCCGCCCAGGAAGGTCA
afsR_R+1576	CATGAAGCTTCG**AGATCT**CCCGCCTGGAGCTCGTC[a]
afsR_F+1562	CATGGAATTCAGGCGGGAGATCTCGCGGTGAAGGC[a]
afsR_R+3390	CATG**AAGCTT**CACACATTCCCCCGTACCGCGA
afsR_R+2649	CATG**AAGCTT**CCCGTGCTACAGCTGCTGCCGGCTC[b]

[#]Restriction sites used in oligos are underlined and in bold. GAATTC, EcoRI; AGATCT, BglII; AAGCTT, HindIII; TCTAGA, XbaI;

[a] A in black shade of oligo afsR_F+1562 was designed to replace C on wild type sequence, to create restriction site BglII with silent mutation. T in black shade of oligo afsR_R+1576 has the same designation.

[b] Stop codon (CTA) in black shade of oligo afsR_R+2649 was designed to replace Trp codon (TGG) on wild type sequence, to create non-sense mutation.

Constructs for creating in-frame deletion mutant

Creation of knock-out mutants is based on pWHM3, which is a multi-copy and unstable *Streptomyces* vector (Vara *et al.*, 1989). See Chapter II and III for details. For the deletion of *afsR*, its upstream region -1383/+6 (using primer pair afsR_LF-1383 and afsR_LR+6) and downstream region +2920/+4367 (using primer pair afsR_RF+2920 and afsR_RR+4367) were amplified by PCR on *S. griseus* IFO13350 genomic DNA. DNA fragments harboring flanking regions were cloned into pWHM3, and subsequently the engineered XbaI site in-between the inserts was used to insert the apramycin resistance cassette *aac(3)IV* flanked by *loxP* recognition sites. The resulting plasmid was called pGWS753. The presence of *loxP* sites allows efficient removal of apramycin resistance cassette by Cre-recombinase, expressed from plasmid pUWL-Cre, to generate a marker-less deletion mutant (Fedoryshyn *et al.*, 2008). To analyze the correctness of the mutant, PCR was done on genomic DNA of strain GAL74, using primer pair afsR_F-153 and afsR_R+2964.

Cloning and expression of afsR, truncated afsR (afsR) from S. griseus*

Primer pairs afsR_F-480 and afsR_R+1576 and afsR_F+1562 and afsR_R+3390 were used to amplify -480/+1576 region and +1562/+3390 region relative to *afsR* from *S. griseus* IFO13350 genomic DNA. The PCR-engineered BglII site allowed cloning of *afsR* and its upstream and downstream sequences as two parts, with introduction of a C → A mutation at nt position +1569 of *afsR*. A 3.9 kb DNA fragment encompassing the entire coding region of *afsR* as well as its putative promoter region and downstream gene *orf4*, was cloned into pHJL401 to generate pGWS781. To express *afsR**, the truncated *afsR*, primers afsR_F+1562 and afsR_R+2649 were used to amplify the +1562/+2649 region relative to *afsR* from *S. griseus* IFO13350 genomic DNA. Primer afsR_R+2649 was designed to introduce replace codon 881 of *afsR* by a stop codon. The approximately 1 kb fragment was cloned as a BglII-HindIII fragment into pGWS781 to replace the terminal part of *afsR* and downstream sequences, to generate pGWS782.

Streptomycin activity assay

To assess the level of streptomycin production, wild-type *S. griseus* IFO13350 and its derivatives were grown on Bennett's maltose agar plates (Hirano *et al.*, 2008) for 5 days, then overlaid with culture of *B. subtilis* as an indicator as described previously (Horinouchi *et al.*, 1984).

Stereo microscopy

Strains were grown on SFM plates for four days at 30°C. The strains were imaged using a Zeiss Lumar V12 stereomicroscope.

HPLC-Q_TOF

Approximately 100 colonies for each strain were spread out on 6,25 ml R5 agar solid medium containing thiostrepton (20 μg/ml), and incubated at 30°C for seven days. Then the spent medium was soaked into 30 ml ethyl acetate overnight. The liquid phase was evaporated, and the extracted compounds re-dissolved in 300 μl ethanol for UHPLC-TOF-MS. UHPLC-TOF-MS analyses were performed on a UHPLC system (Ultimate 3000, ThermoScientific, Germany) coupled to an ESI-Q-TOF spectrometer (micrOTOF-QII, Bruker Daltonics, Germany). Detection was done in the negative mode. The *m/z* range was set to be 100–600mz. The ESI conditions were as follows: capillary voltage of 3500V, source temperature of 250°C, dry gas flow 10 ml min^{-1}. For internal calibration, a 10 mM solution of sodium formate (Fluka, Steinheim, Germany) was infused. UHPLC-TOF-MS chromatograms were obtained using a 150mm× 2.1 mm i.d., 2.6 micron Kinetex C18 UPLC column (Phenomenex, USA) in gradient mode at a flow rate of 0.3 ml min^{-1} with the following solvent system: (A) 0.1 vol% FA(formic acid) in water; (B) 0.1 vol% FA in methanol. Analysis began with gradient of 5% to 95% B over 14 min, followed by an isocratic step of 95 B from 14 to 15 min and a re-equilibration step of 5% B from 15 to 17 minutes. The temperature was maintained at 30 °C, and the injection volume was 3 μl.

Computer analyses

DNA and protein databank searches were performed using and StrepDB page services (http://strepdb.streptomyces.org.uk/).

RESULTS AND DISCUSSION

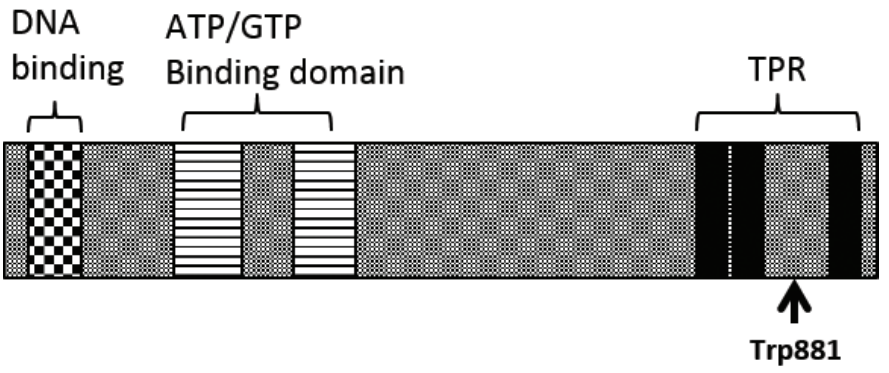
S. griseus AFN contains a mutation in *afsR*

The genome of the spontaneous A-factor nonproducer strain *S. griseus* AFN (Biró *et al.*, 2000) was sequenced by Illumina paired end sequencing and compared to wild-type *S. griseus* IFO13350 (Ohnishi *et al.*, 2008) to identify the mutations in *S. griseus* AFN relative to its parent strain *S. griseus* NRRL B2682. Around 100 single nucleotide permutations (SNPs) were found between AFN and the published sequence of wild-type *S. griseus* IFO13350. Of these, most were also found in *S.* griseus NRRL B2682. Importantly, one mutation that was specific to *S.* griseus mutant AFN was a nonsense mutation in *afsR*, which caused a premature translational stop by mutating the Trp881 codon TGG to a TAG stop codon. AfsR is a DNA binding protein that is conditionally needed for development and antibiotic production of *S. griseus* and *S. coelicolor* (Umeyama *et al.*, 1999; Floriano and Bibb, 1996).

Gene organization around *afsR* region in *S. griseus* and *S. coelicolor*

AfsR is phosphorylated by phosphokinase AfsK at Ser and Thr residues in various *Streptomyces* spp., including *S. coelicolor*, *S. lividans* and *S. griseus* (Atsushi *et al.*, 1994; Hong *et al.*, 1991; Umeyama *et al.*, 1999). AfsR contains a DNA-binding and an ATPase domain, which are both required for AfsR function (Lee *et al.*, 2002) (Fig. 1A). The C-terminal domain of AfsR contains three so-called tetratricopeptide repeats (TPR) (Fig. 1A), which are important for mediating protein-protein interactions (Blatch and Lässle, 1999). The gene organization around *afsR* in *S .coelicolor* and *S. griseus* is very different (Fig. 1B). In both species, a small ORF lies downstream of *afsR*. In *S. coelicolor* this *afsS*, for a 63 aa activator of secondary metabolism (Tanaka *et al.*, 2007), while the function of the putative 58 aa protein encoded by the downstream ORF in *S. griseus* is unknown. Furthermore, while in *S. coelicolor afsK* and *afsR* lie 1 kb away from each other, in *S. griseus* these genes are spaced by some 6 kb.

1A



1B

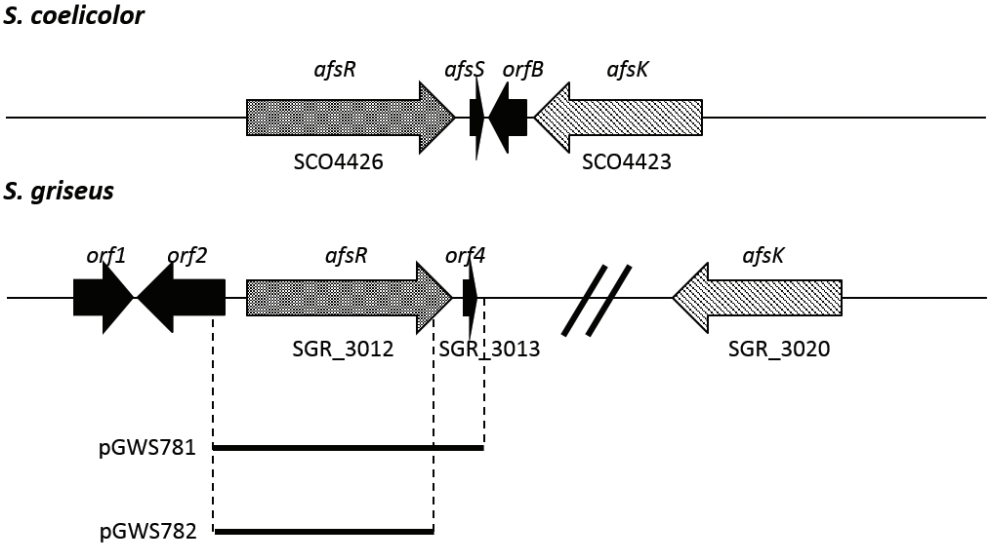


Figure 1. Protein domain structure of AfsR and gene organization of the *afsR* locus.
(A) Domain structure of the AfsR protein. AfsR is a SARP-family regulator, with an N-terminal DNA binding domain, an ATP binding motif in the middle, and three tetratricopeptide repeats at the C-terminal end of the protein. The non-sense mutation at position of Trp881 is indicated. **(B)** Genetic organization of the region around *afsR* and *afsK* in *S. coelicolor* and *S. griseus*. Note that in *S. coelicolor afsR* and *afsK* are closer (spacing 1 kb encompassing two ORFs between the genes) than in *S. griseus* (6 kb spacing encompassing six ORFs). The inserts of plasmids pGWS781 (harboring *S. griseus afsR* and the small downstream ORF) and pGWS782 (harboring truncated *afsR**) are indicated by a horizontal line.

Deletion of *afsR* conditionally accelerates development of *S. griseus*

An *afsR* null mutant was created in *S. griseus* IFO13350 by deleting the +7/+2919 bp region relative to *afsR* (see Materials and Methods section). After replacement of the *afsR* gene by the apramycin resistance cassette using knock-out construct pGWS753, the apramycin resistance cassette was excised following expression of the Cre recombinase from plasmid pUWL-Cre. The resulting marker-less deletion mutant was designated GAL74. Previously, it was published that an *afsR* null mutant of *S. griseus* had a bald phenotype on YMPD media containing 1.5% glucose (Umeyama *et al.*, 1999). However, this mutant is not available any more. In contrast, at least in our hands, deletion of *afsR* in *S. griseus* IFO13350 did not cause major developmental defects when the strain was grown on YMPD media containing 1.5% glucose, and the mutant sporulated as well as the parental strain after 7 days of incubation (Data not shown).

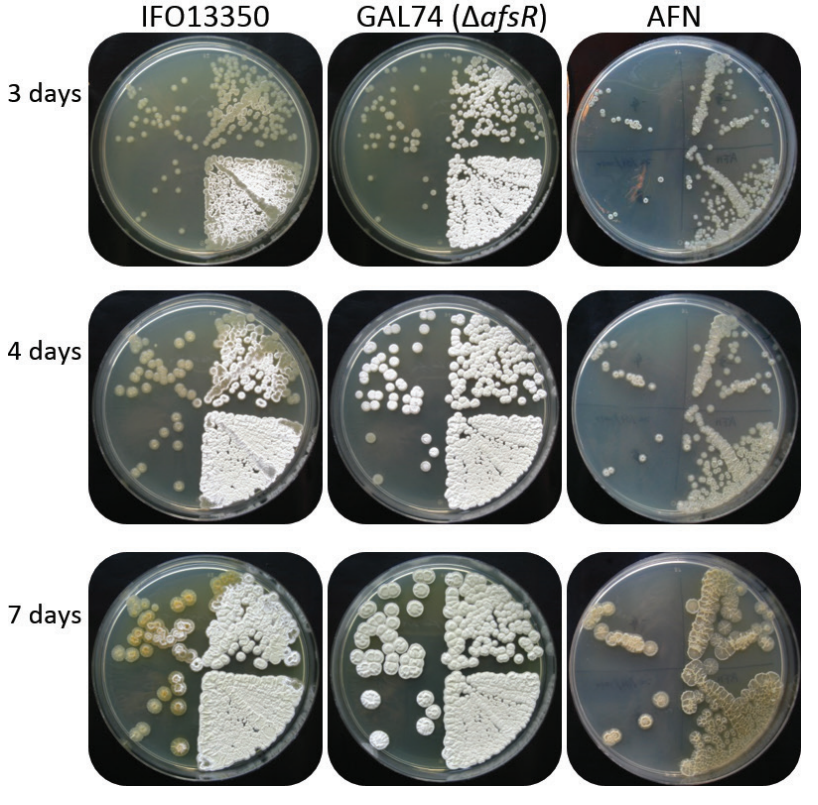


Figure 2. Growth of wild type *S. griseus* IFO13350, its *afsR*-null mutant (GAL74) and *S. griseus* B2682 AFN on R5 medium. Dilutions of spore suspensions (for *S. griseus* IFO13350 and *afsR* null mutant GAL74) or liquid-grown mycelium (for nonsporulating mutant *S. griseus* AFN) were plated onto R5 agar plates. Plates were photographed after 3 days, 4 days or 7 days of growth at 30 °C.

Interestingly, when the same dilutions were plated on R5 agar plates, ranging from a density of a few to some 1000 colonies per quarter agar plate, significant differences were observed between GAL74 and the parental strain. Consistent with previous observations (Biró et al. 2000), *S. griseus* mutant AFN failed to form aerial hyphae irrespective of incubation time and density of inoculation (Fig. 2, right column). After three days of growth, at high density both wild-type *S. griseus* and its *afsR* null mutant produced aerial hyphae. However, at lower densities, single colonies of GAL74 formed aerial hyphae much earlier than the parental strain, and at the lowest dilution in fact the parental strain produced very few aerial hyphae even after 7 days, while those of GAL74 had fully developed. Taken together, these data show that the absence of *afsR* conditionally accelerates the developmental program in *S. griseus*, in a cell density-dependent manner.

Expression level of AfsR affects growth and development of *S. griseus*

To study the effect of AfsR on development, construct pGWS781 (Fig. 1B), which harbors *afsR* with 480 bp promoter region and downstream sequences including the downstream located ORF4 (see Fig. 1B), was transformed to *S. griseus* IFO13350 and its *afsR* null mutant GAL74, resulting in strains GAL76 and GAL79, respectively. Similarly, construct pGWS782 (Fig. 1B), which expresses truncated AfsR* (which has codon Trp881 changed to a stop codon), was also introduced into *S. griseus* IFO13350 and GAL74 to generate strains GAL77 and GAL80, respectively. *S. griseus* IFO13350 and GAL74 containing empty plasmid were used as controls. Thiostrepton (20 μg/ml) was added to the media to maintain constructs in transformants. All constructs were based on plasmid pHJL401, which is a low-copy number vector (around 5-10 copies per chromosome) in *Streptomyces*.

On YMPD plates with glucose (1.5% w/v) and with thiostrepton as selective marker for the plasmids, all strains fully developed when grown at high density. Differences in growth were mainly observed in the quadrants with fewer colonies. Introduction of additional copies of *afsR* or *afsR** into wild-type *S. griseus* IFO13350 (GAL76 and GAL77, respectively) promoted aerial hyphae formation in the quadrant with around a hundred colonies, while strains harboring the control plasmid showed very limited aerial hyphae formation (Fig. 3A). At the next lowest dilution, GAL76 and GAL77 entered aerial growth where the control strain grew vegetatively (Fig. 3A). However, no effect of the introduction of pHJL401/*afsR* or pHJL401/*afsR** was seen in the *afsR* null mutant. Similarly, on R5 agar plates with thiostrepton, in the quadrant with

around a hundred colonies, and around the edges of the high density patches, introduction of *afsR* or to a lesser extent *afsR** into wild-type cells accelerated aerial hyphae formation in colonies grown in close proximity (Fig. 3B). Again, this effect was far less strong in *afsR* mutant colonies.

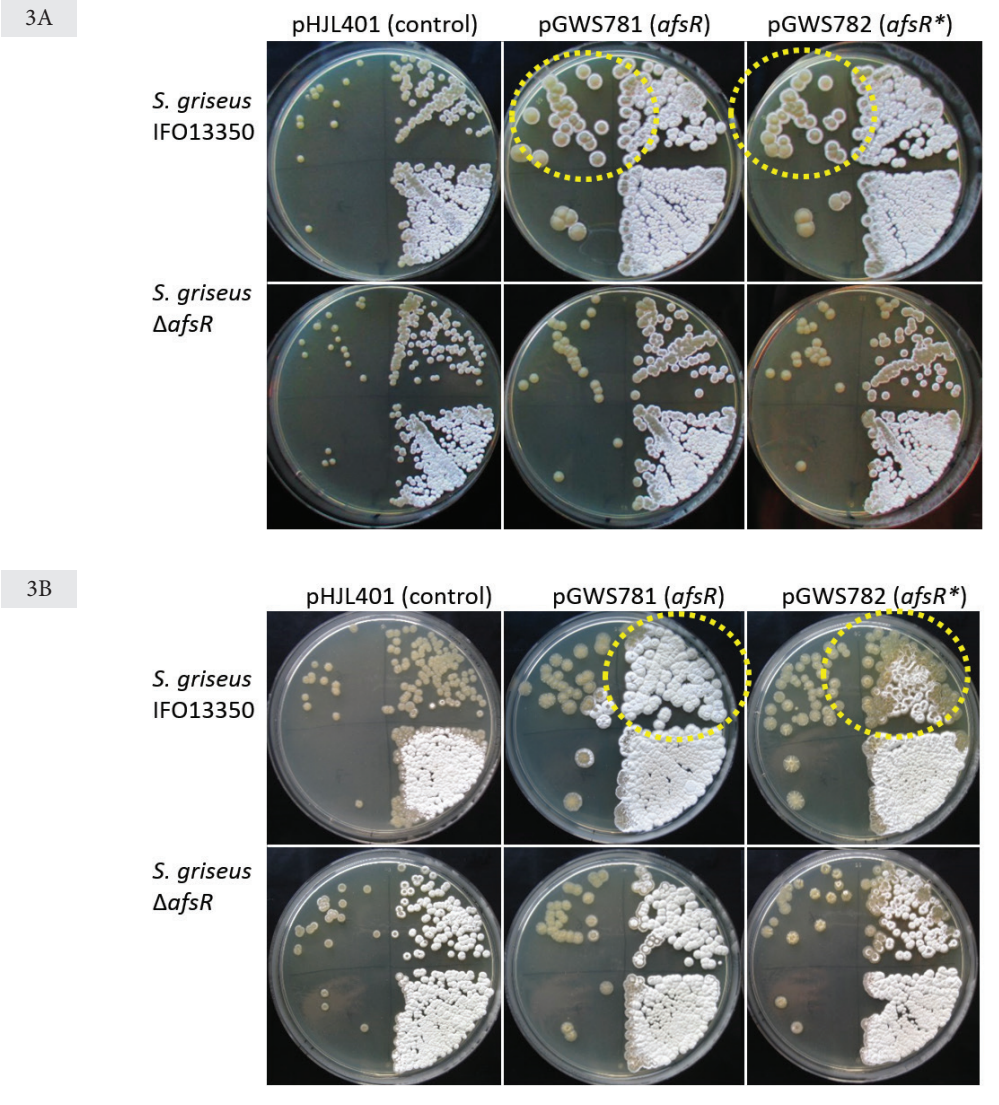


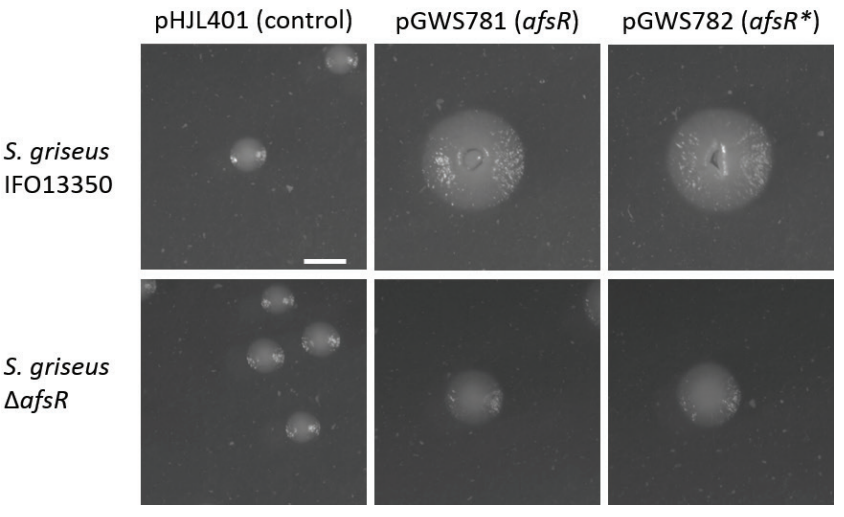
Figure 3. Effect of *afsR* and *afsR on development of *S. griseus* IFO13350.** Overview of whole plates on YMPD (1.5% glucose) agar plates **(A)** and on R5 agar plates **(B)**. Dilutions of spore-preps of *S. griseus* IFO13350 or its *afsR* null mutant with either control plasmid (pHJL401), plasmid pGWS781 (pHJL401 + *afsR*) or pGWS782 (pHJL401 with *afsR**) were plated onto YMPD (1.5% glucose) and R5 agar plates with thiostrepton (20 µg/ml). Photos were taken after 7 days incubation at 30 °C.

Correlation between AfsR and colony size

While differences in the timing of development were significant yet relatively small, a striking difference was observed in terms of colony size irrespective of culturing conditions. Colonies harboring control plasmid pHJL401 were very similar in size. However, introduction of pGWS781, which harbors *afsR*, in wild-type cells led to an approximately 10-fold increase in colony size, and a similar increase was seen for pGWS782 harboring *afsR** (Fig. 4A). Interestingly, introduction of either plasmid in the *afsR* null mutant GAL74 had a very small effect on colony size (Fig. 4A). This suggests that the copy number of *afsR* and/or that of the downstream gene, which is higher in the wild-type strain than in the *afsR* mutant, plays an important role in determining colony size, and that *afsR** is equally active in this respect as wild-type *afsR*. Since the downstream gene (SGR_3013; Fig. 1B) is absent from the construct expressing *afsR**, it is unlikely that this gene plays a major role in determining colony size.

To test the stability of the plasmids, all transformants were grown as a confluent lawn on agar plates in the absence of thiostrepton, replicated twice to nonselective media, and then spores were harvested and plated for single colonies on agar plates with or without thiostrepton, and resistance to thiostrepton assessed. Interestingly, control plasmid pHJL401 was lost almost completely by wild-type cells after two rounds of nonselective growth. However, the plasmid harboring *afsR* or *afsR** was maintained by around 70% and 27% of the cells, respectively (Fig. 4B). When the same experiment was done for *afsR* null mutant GAL74, all three plasmids were rapidly lost (Fig. 4B). These data are consistent with growth promotion by additional copies of *afsR* and to a lesser extent *afsR**, as this would select for maintenance of the plasmid. The lack of such an effect in *afsR* null mutants (see Fig. 3) explains the loss of the pHJL401/*afsR* and pHJL401/*afsR** plasmids in this genetic background.

4A



4B

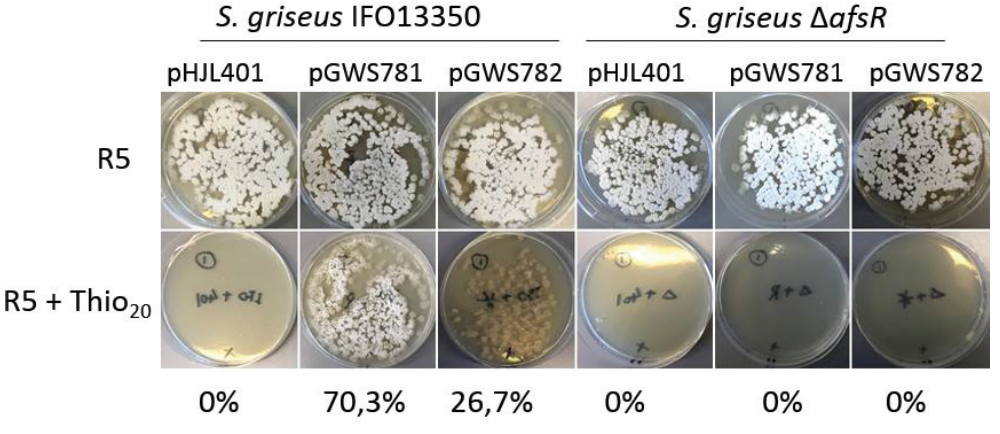


Figure 4. Effect of *afsR* and *afsR on colony size and plasmid stability of *S. griseus* IFO13350.** **(A)** Stereomicrographs of representative colonies to show the effect on colony size. Dilutions of spore-preps of *S. griseus* IFO13350 or its *afsR* null mutant with either control plasmid (pHJL401), plasmid pGWS781 (pHJL401 + *afsR*) or pGWS782 (pHJL401 with *afsR**) were plated onto R5 agar plates with thiostrepton (20 μg/ml). Photos were taken after 7 days incubation at 30 °C. Bar, 2 mm. **(B)** Detection of plasmid maintaining in transformants of wild type *S. griseus* IFO13350 and in *afsR* null mutants. Percentage of cells still containing plasmid after two rounds of non-selective growth were indicated at bottom.

AfsR enhances streptomycin production

It was shown previously that over-expression of *afsR* in different Streptomyces species promotes antibiotic production (Kim *et al.*, 2012; Stutzman-Engwall *et al.*, 1992; Floriano and Bibb, 1996). Therefore, we compared the effect of *afsR* copy number on streptomycin production in solid-grown cultures, using *Bacillus subtilis* as the indicator strain. Streptomycin production was measured as the size of the inhibition zones around the colonies. Consistent with such a stimulation of antibiotic production, extra copies of either *afsR* or *afsR** enhanced streptomycin production in *S. griseus* IFO13350 (Fig. 4). Interestingly, introduction of a plasmid with *afsR** (which also lacks the downstream gene SGR_3013) stimulated streptomycin production more strongly than a plasmid with wild-type *afsR* (Fig. 4). Deletion of *afsR* did not cause a noticeable change in streptomycin production, with the size of the inhibition zones of strain GAL78 (*afsR*-null mutant with empty plasmid) being comparable to that of wild-type *S. griseus* IFO13350 with empty plasmid (Fig.5). This is in line with earlier observations (Umeyama *et al.*, 1999). Complementation of the *afsR* null mutant with *afsR* or *afsR** only slightly enhanced streptomycin production (Fig. 5).

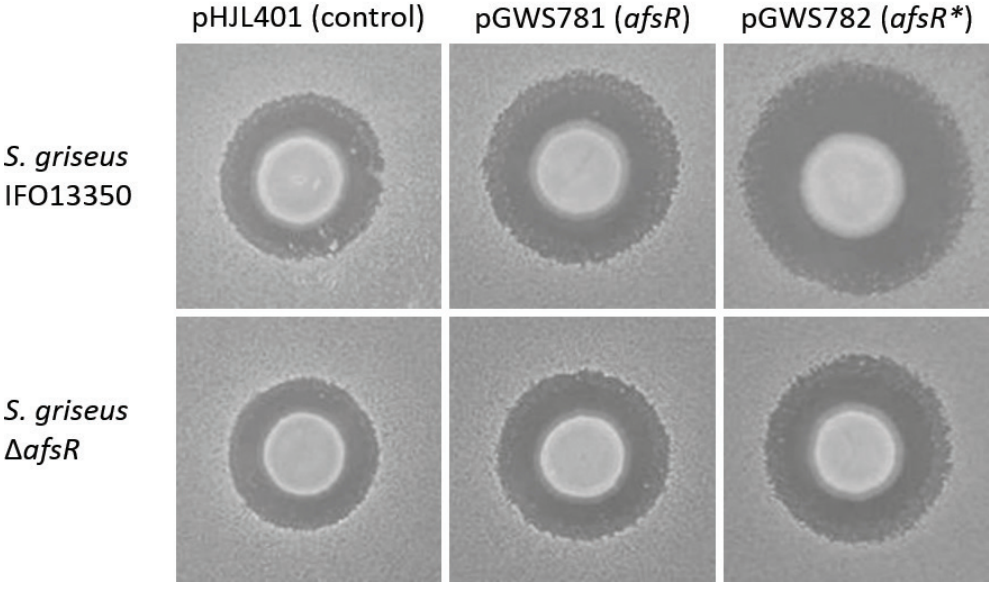


Figure 5. Effect of *afsR* and *afsR on streptomycin production.** Strains of *S. griseus* were inoculated on Bennett maltose agar using a toothpick and overlaid with soft agar containing *B. subtilis* after 5 days. For details on strains see Figure 3.

A-factor production is apparently not influenced by *afsR*

S. griseus B2682 AFN, which is a spontaneous A-factor non-producer, has a bald phenotype and fails to produce streptomycin, in accordance with a role of A-factor in signaling the onset of morphological and chemical differentiation (Birkó *et al.*, 2007). To establish whether the *afsR** mutation is the cause of the reduced A-factor production, the amount of A-factor produced by the different strains was determined using HPLC-Q-TOF. The acquisition time of A-factor was at approximately 11.5 min, giving a peak with an m/z of 241 Da in negative ion mode (Fig. 6). Perhaps surprisingly, we failed to detect major differences in A-factor production, although the peak in the sample corresponding to the largest inhibition zones (strain GAL77, IFO13350 + *afsR**) was somewhat larger than those for other strains. These data suggest that *afsR* or its mutant version *afsR** does not have a major impact on A-factor production (Fig. 6).

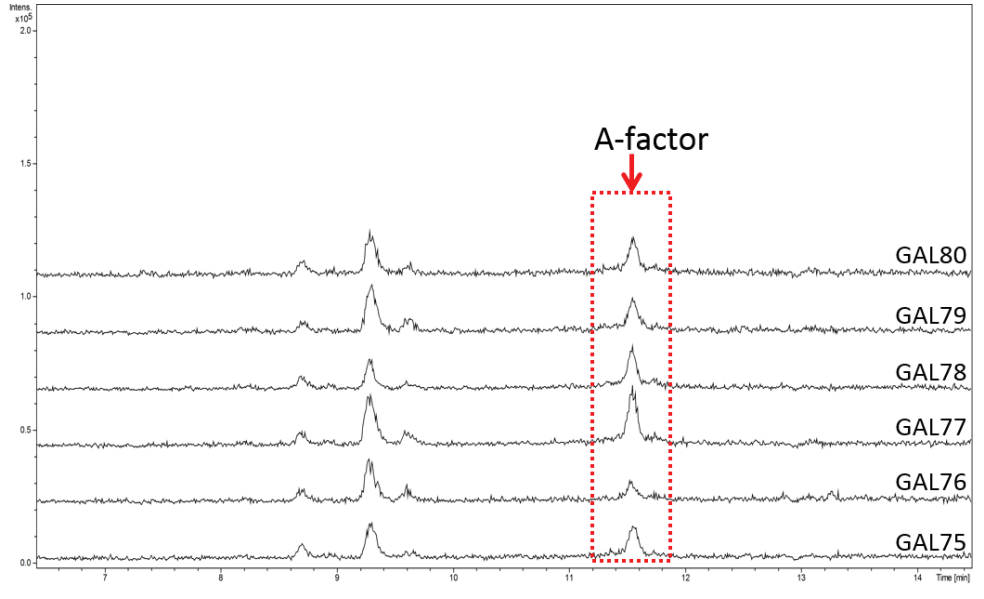


Figure 6. UHPLC-TOF mass spectra of A-factor produced by strains of *S. griseus*. GAL75, GAL76 and GAL77 are *S. griseus* IFO13350 harboring pHJL401, pHJL401/afsR and pHJL401/afsR*, respectively; GAL78, GAL79 and GAL80 are the *afsR* null mutant harboring pHJL401, pHJL401/*afsR* and pHJL401/*afsR**, respectively.

DISCUSSION

AdpA is a master transcriptional activator in *Streptomyces*, which controls many genes involved development and secondary metabolite production (Higo *et al.*, 2012; Ohnishi *et al.*, 2005). The autoregulatory hormone-like molecule A-factor binds to the ArpA protein, so as to allow activation of the AdpA-dependent regulatory network (Ohnishi *et al.*, 2005). An unsolved mystery is a spontaneous mutant of *S. griseus* NRRL B2682 that has the ability to produce A-factor, but fails to produce enough of the molecule to allow development and antibiotic production to proceed. None of the genes relating to A-factor production carries a mutation. In fact, surprisingly, expression of the large signaling protein Factor C, restored development to the *S. griseus* AFN mutant. This suggested that the AFN mutation should be located in a novel gene of the A-factor pathway. Thus, identification of the mutation that causes the AFN mutation should provide new insight in the very important A-factor regulatory cascade.

This work shows that one of the mutations in fact causes truncation of AfsR, a regulator of development and antibiotic production. But is the nonsense mutation in *afsR* really the main cause of the non-sporulating phenotype of *S. griseus* AFN? The answer is probably negative. While the AFN mutant produced minute amounts of A-factor, and failed to produce aerial hyphae or streptomycin, strain GAL80 (which essentially has wild-type *afsR* replaced by the truncated *afsR** variant found in AFN) showed normal development and streptomycin production. This was explained by the fact that A-factor production in GAL80 was comparable to that of the wild-type strain. Therefore, it is highly unlikely that the nonsense mutation in *afsR* is the (sole) cause of the AFN phenotype. The many mutations in the AFN mutant, and the fact that it was made in a background of *S. griseus* NRRL B2682 and not of the sequenced IFO13350, make looking for the AFN mutation looking for the proverbial needle in the haystack.

Nevertheless, our data did establish a role of AfsR in the control of development, colony size and streptomycin production in *S. griseus*. The *afsR* expression level positively correlates to colony size, which in turn is directly proportional to streptomycin production. However, in *S. coelicolor*, AfsR has been shown to have a negative effect on colony size, with extra copies of *afsR* reducing colony size on various solid media (Floriano and Bibb, 1996).

Mutants of *S. coelicolor* that lack the *ssgB* gene, which encodes a positive regulator of *Streptomyces* cell division during sporulation (see *e.g.* Chapter II),

have a large colony variant (LCV) phenotype, with colonies more than twice the size of wild-type colonies (Keijser *et al.*, 2003). The *ssgB* mutant colonies produce a very large aerial mycelium, and these mutants apparently lack the signal that enforces aerial hyphae to stop growing, which is a prerequisite for the onset of sporulation-specific cell division (Jakimowicz and van Wezel, 2012). In terms of a correlation between colony size and antibiotic production, deletion or mutation of the antibiotic regulatory gene *absB* resulted in an SCV phenotype in *S. coelicolor* (Price *et al.*, 1999; Adamidis and Champness, 1992), while microcystin production by *Microcystis* positively correlated to colony size (Kurmayer *et al.*, 2003). Small colony variants (SCV) also occur in other bacteria, including *Brucella, Burkholderia*, *Escherichia, Lactobacillus, Neisseria, Pseudomonas* and *Salmonella* (Proctor *et al.*, 2006; Morris *et al.*, 1943; Rohde, 1999; Morton and Shoemaker, 1945; Kopeloff, 1934; Colwell, 1946; Häussler *et al.*, 2003; Hall and Spink, 1947). Changing to SCV allows pathogens to escape the host defense system from antibiotics and persistence in mammalian cells (Proctor *et al.*, 2006; von Eiff *et al.*, 2001). Elucidation of the molecular background of the LCV (and SCV) phenotype of colonies, and the role of *afsR* in this phenomenon, should allow us to better understand the factors that control growth and antibiotic production of streptomycetes.

In terms of development, it has been observed previously that deletion or enhanced expression of *afsR* blocks development of *S. griseus* on media containing glucose as sole carbon source (Umeyama *et al.*, 1999). However, in our hands, deletion of *afsR* did not have a major effect on growth or development, while introduction of additional copies of *afsR* in wild-type cells accelerated development. Unfortunately, the original *afsR* mutant is lost and we cannot compare our mutant with those produced by Umeyama and colleagues. Our study revealed that AfsR plays a role in the control of growth and streptomycin production, with a promising stimulation of streptomycin levels by AfsR and particularly of AfsR*. Clearly, more extensive system biology approaches are required to decipher the AfsR regulon and analyze its yet ambiguous role in the control of growth and antibiotic production in streptomycetes.