Involvement of \textit{amfC} in physiological and morphological development in \textit{Streptomyces coelicolor} A3(2)

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\textit{amfC} plays a regulatory role in aerial mycelium formation in \textit{Streptomyces griseus} and is distributed widely among \textit{Streptomyces} species. Disruption of the chromosomal \textit{amfC} gene in \textit{Streptomyces coelicolor} A3(2) severely reduced formation of aerial hyphae, indicating that \textit{amfC} is important in morphological development. In addition, the disruption caused \textit{S. coelicolor} A3(2) M130 to produce much less actinorhodin, and to produce undecylprodigiosin at a later stage of growth, indicating that \textit{amfC} also regulates secondary metabolism. S1 nuclease mapping showed that transcription of actII-ORF4, the pathway-specific transcriptional activator in the act gene cluster, was greatly reduced in the \textit{amfC} disruptants. The defect in secondary metabolite formation was suppressed or overcome by a mutation in \textit{sre-1}. Consequently, an \textit{amfC}-disrupted strain derived from \textit{S. coelicolor} A3(2) M145, an actinorhodin-overproducing strain due to the \textit{sre-1} mutation, still produced a large amount of actinorhodin. Extra copies of \textit{amfC} in strains M130 and M145 did not change spore-chain morphology or secondary metabolite formation. However, the spores in these strains remained white even after prolonged incubation. Since only spore pigmentation was affected, all known \textit{whi} genes, except \textit{whiE}, responsible for the polyketide spore pigment formation, were assumed to function normally. S1 nuclease mapping showed that transcription of \textit{whiE}P1, one of the promoters in the \textit{whiE} locus, was reduced in \textit{S. coelicolor} A3(2) containing extra copies of \textit{amfC}. Introducing \textit{amfC} into several other \textit{Streptomyces} species, such as \textit{Streptomyces lividans}, \textit{Streptomyces lavendulae} and \textit{Streptomyces lipmanii}, also abolished spore pigment formation. An increase in the amount of AmfC appeared to disturb the maturation of spores.

Keywords: \textit{Streptomyces coelicolor} A3(2), \textit{amfC}, aerial mycelium formation, antibiotic production, polyketide spore pigment

INTRODUCTION

Gram-positive soil bacteria of the genus \textit{Streptomyces} are characterized by complex morphological differentiation resembling that of filamentous fungi, and by the ability to produce a wide variety of secondary metabolites, including antibiotics (Chater, 1984, 1989). In \textit{Streptomyces griseus}, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) acts at an extremely low concentration as a switch for streptomycin production, streptomycin resistance, yellow pigment production and aerial mycelium formation (Horinouchi & Beppu, 1992, 1994). Because of the essential role of A-factor in physiological and morphological development, A-factor-deficient mutants of \textit{S. griseus} cannot form streptomycin or aerial mycelium (Horinouchi, 1996). Screening a DNA library of chromosomal genes from the wild-type strain for DNA fragments that restored aerial mycelium formation in the A-factor-deficient mutant strain HH1 has led to the isolation of an operon and two genes: \textit{amfR}–\textit{amfA}–\textit{amfB} (Ueda et al., 1993, 1998), \textit{orf1590} (Babcock & Kendrick, 1990; Ueda et al., 1993) and \textit{amfC} (Kudo et al., 1995). \textit{amfC} encodes a 218 aa protein that does not restore A-factor or streptomycin production, indicating that this gene acts in aerial mycelium formation independently of its secondary metabolic function (Kudo et al., 1995). Disruption of the...
chromosomal *amfC* in wild-type *S. griseus* severely reduced the abundance of spores due to inefrquent sporulation. Nucleotide sequences homologous to *amfC* are distributed in all 12 *Streptomyces* species tested (Kudo et al., 1995), which suggests a common role of *amfC*. An AmfC homologue (222 aa) in *Streptomyces coelicolor* A3(2) shows 60% identity in amino acid sequence to AmfC in *S. griseus*.

These observations prompted us to determine the role of *amfC* in *S. coelicolor* A3(2), the most intensively studied streptomycete. Our results show that *amfC* plays the same role in aerial mycelium formation as in *S. griseus*. In addition, we found that disruption of *amfC* severely reduced actinorhodin production. Overexpression of *amfC* abolished spore pigment formation almost completely. This paper describes the phenotypes of *amfC* disruptants and strains containing extra copies of *amfC*. It also describes transcription in these strains of *wbiE*, responsible for spore pigment formation, and *actII-ORF4*, responsible for actinorhodin production.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** *S. griseus* IFO 13350 (Hara et al., 1983) was obtained from the Institute of Fermentation, Osaka, Japan (IFO). *S. coelicolor* A3(2) strains M130 (*hisA1 uraA1 SCP1− SCP2∗) and M145 (see-1 SCP1− SCP2∗) and *S. lividans* strains TK21 and TK24 were obtained from D. A. Hopwood, John Innes Research Centre, Norwich, UK (Hopwood et al., 1985). A pUC19 derivative containing the whole *amfC* sequence in a 2.7 kb BamHI fragment (Kudo et al., 1995) was used as a source of *amfC* from *S. coelicolor* A3(2) M130. Other *Streptomyces* strains were obtained from IFO. Plasmids pJ1486, with a copy number of 40–100 per genome (Ward et al., 1986), and pKU209, with a copy number of 1–2 (Kakinuma et al., 1991), were used as *Streptomyces* cloning vectors. DNA was manipulated in *Escherichia coli*. SFM and TSB media were described by Floriano & Bibb (1987). *Actinomycete* strains TK21 and TK24 were obtained from IFO. Plasmids pIJ486, with a copy number of 40–100 per genome (Ward et al., 1985), and pIJ485 (see-1 SCP1− SCP2∗) were obtained from D. A. Hopwood, John Innes Research Centre, Norwich, UK (Hopwood et al., 1985). A pUC19 derivative containing the whole *amfC* sequence in a 2.7 kb BamHI fragment (Kudo et al., 1995) was used as a source of *amfC* from *S. coelicolor* A3(2) M130. Other *Streptomyces* strains were obtained from IFO. Plasmids pJ1486, with a copy number of 40–100 per genome (Ward et al., 1986), and pKU209, with a copy number of 1–2 (Kakinuma et al., 1991), were used as *Streptomyces* cloning vectors. DNA was manipulated in *Escherichia coli*. SFM and TSB media were described by Floriano & Bibb (1996) and Onaka et al. (1998), respectively. Histidine (50 mg l−1) and uracil (7.5 mg l−1) were added when necessary.

**Recombinant DNA studies.** Restriction enzymes, T4 DNA ligase and other DNA-modifying enzymes were purchased from Takara Shuzo, [α-3²P]dCTP (110 TBq mmol−1) for DNA labelling with the Takara BacBest DNA labelling system and [γ-3²P]ATP (220 TBq mmol−1) for end-labeling at 5′ ends with T4 polynucleotide kinase were purchased from Amersham. DNA was manipulated in *Streptomyces* as described by Hopwood et al. (1985) and in *E. coli* as described by Maniatis et al. (1982).

*amfC* was cloned in pKU209 and pJ1486 as follows: an 897 bp *EcoT14I−AccIII* fragment containing *amfC* and its promoter (see below) was flush-ended with the Klenow fragment and inserted into the *HinCII* site of pUC19. The recombinant pUC19 plasmid was digested with HindIII and flush-ended with the Klenow fragment. An EcoRI linker was then attached to the ends and the *amfC* sequence, excised as an EcoRI fragment, was inserted into the *EcoRI* sites of pJ1486 and pKU209 to give pJ1486-amfC and pKU209-amfC, respectively.

**Gene disruption.** To disrupt the chromosomal *amfC* in *S. coelicolor* A3(2), we used single-stranded M13 phage DNA as described by Hillemann et al. (1991). For construction of an M13 recombinant plasmid containing a neomycin phospho-transferase (*neo*) gene, a 1.32 kb *SmaI−HindIII* fragment from Tn5 (Beck et al., 1982) was first cloned in pUC19. A 307 bp *SmaI−PmaCI* fragment encoding the internal part (Arg97−Val108) of AmfC was then inserted into the *SmaI* site. Single-stranded DNA was prepared from *E. coli* JM109 and introduced into *S. coelicolor* A3(2) strains M130 and M145 by transformation. After neomycin (20 µg ml−1)-resistant transformants had been selected, they were screened for true disruptants by Southern hybridization of their BamHI-digested chromosomal DNA with the *neo* sequence and the 307 bp *SmaI−PmaCI* fragment as probes.

**Electron microscopy.** Spores and hyphae of *S. coelicolor* A3(2) strains grown at 28 °C for 7 d on R2YE agar were examined by scanning electron microscopy (Takamatsu et al., 1976). To prepare specimens, agar blocks were fixed with 2% osmium tetroxide for 40 h and then dehydrated by air-drying. Each specimen was sputter-coated with platinum/gold and examined with a Hitachi S4000 scanning electron microscope.

**Assay of actinorhodin and undecylprodigiosin.** Actinorhodin was detected visually by its blue colour. For quantification, actinorhodin production in R2YE liquid medium was estimated from the A450 of the culture broth at pH 12, as described by Onaka et al. (1998). Undecylprodigiosin in mycelium was measured (Onaka et al., 1998) by extracting the pigments with methanol from mycelium grown at 28 °C for 7–9 d on a cellophane sheet laid on the surface of R2YE agar medium. After concentration of the extract by evaporation, the orange pigment at Rf 0.35 was separated out by thin-layer (Whatman KC18F reverse-phase plate) chromatography with 100% methanol as the solvent and eluted with the same solvent. The amount was calculated from the A433 of the eluate. Undecylprodigiosin shows absorption maxima at 533 and 468 nm at acidic and alkaline pH, respectively.

**S1 nuclease mapping.** RNA was isolated from mycelium grown at 30 °C for 2–7 d on cellophane placed on the surface of R2YE or SFM agar, as described previously (Horinouchi et al., 1987). [³²P]-labelled probes were prepared by PCR with primers I and II and a template of strain M145 chromosomal DNA. When the 5′ end of primer II was 5′-rP-labelled with T4 polynucleotide kinase, the PCR product could be used as the probe. To determine the transcriptional start point of *amfC*, primer I (5′-TAGGGTGGAGGGCCGCCTGCT-3′; corresponding to −155 to −132, taking the A residue of the ATG start codon of *amfC* as +1) and primer II (5′-ACCCACCGGCGCCCGACCTGCT-3′; corresponding to +96 to +73) were used. For *actII-ORF4*, primer I (5′-AATTTTTTGAATAGGAGATCGCGTTG-3′; corresponding to −108 to −82, taking the A residue of the ATG start codon of *actII-ORF4* as +1; Fernández-Moreno et al., 1991) and primer II (5′-CGAGACCGCGCCCGCGGTTGCGGATC-3′; corresponding to +358 to +333) were used. For *wbiE*, primer I (5′-GCTTACCGGCTTAACCTCC-3′; corresponding to −147 to −127, taking the A residue of the ATG start codon of *wbiE* as +1; Kelemen et al., 1998) and primer II (5′-GTCCGTATAGGAGGAGACC-3′; corresponding to +26 to +6) were used. For *hrdB*, primer I (5′-GGCCGGAGTACGAAGTTGATACCGTCTGTTTTATCC-3′; corresponding to −279 to −245, taking the first residue of the GTG start codon of *hrdB* as +1; Butner et al., 1990) and primer II (5′-AGGCCGGCGACCAGCTATCGCGCGGACTGCCCC-3′; corresponding to +87 to +121) were used. Marker 10 (pBR322/MspI digest; Nippon Gene) was used to provide size markers. For high-resolution S1
RESULTS
Phenotypes of amfC mutants derived from S. coelicolor A3(2)

(i) Construction of amfC disruptants from strains M130 and M145. To examine the function of amfC, we disrupted the chromosomal amfC gene of S. coelicolor A3(2) M130 by using the neomycin resistance (neo) gene as a selection marker and the amfC sequence truncated at both ends to provide homology for integration of a non-replicating circular molecule by a single cross-over and, as a result of which, to disrupt the gene. True disruptants were selected by Southern hybridization with the neo and truncated amfC sequences as probes (data not shown). This procedure yielded four amfC disruptants (M130ΔamfC). We also generated eight amfC disruptants (M145ΔamfC) from a prototrophic strain, M145. The mutation did not cause any defects in growth, as determined by measuring cell mass in liquid culture or the diameters of colonies growing on solid medium.

(ii) Sparse aerial mycelium formation by amfC disruptants. Both mutants M130ΔamfC and M145ΔamfC formed very few spores on R2YE medium. Fig. 1a shows delayed and infrequent sporulation by M130ΔamfC. The parental strains M130 and M145 began to form aerial hyphae at 3 d after inoculation, whereas aerial hyphae of strains M130ΔamfC and M145ΔamfC appeared at 5 d. Spores in the sporulating area of these mutants were less abundant, although the shape and size of spores of the mutants were indistinguishable from those of the parental strain (Fig. 1c). The delayed and reduced sporulation of M130ΔamfC and M145ΔamfC was also observed on media containing maltose, mannitol or glycerol in place of glucose, in contrast to the situation with many bld mutants whose aerial mycelium formation depends on the carbon source (Merrick, 1976; Chater, 1984). The effect of the amfC disruption on aerial mycelium formation was also seen on R2, SFM and TSB media. Introduction of amfC on a low-copy-number plasmid, pKU209 (plasmid pKU209-amfC) into these mutants restored the defect (data not shown), although a difference in spore pigment formation between the parental strain and these amfC disruptants was noted (see below). These observations indicated an important, but not essential, role for amfC in aerial mycelium formation.

(iii) Effects of amfC mutation on secondary metabolism in strain M130. Strain M130ΔamfC produced almost no blue pigments on R2YE medium (Fig. 1a) as well as R2YE medium containing maltose, mannitol or glycerol.

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**Fig. 1.** Phenotypes of S. coelicolor A3(2) strains M130 and M145 containing the disrupted amfC gene. (a) The parental strain M130 (as a control) and mutant M130ΔamfC were inoculated on R2YE agar and grown for the indicated time (in days) at 30 °C. Mutant M130ΔamfC formed sparse aerial hyphae at 5 d, whereas the parent formed aerial hyphae at 3 d. The blue colour in the centre of the colony of the parent at 5 d is due to actinorhodin produced. Actinorhodin production by the parent was apparent at 4 d, whereas almost no pigment was produced by mutant M130ΔamfC. (b) S. coelicolor A3(2) strains M145 and M145ΔamfC produced almost the same amount of actinorhodin on R2YE agar. The photograph was taken after 7 d growth at 30 °C. Strain M145 forms abundant grey spores, whereas the mycelium of mutant M145ΔamfC looks blue because of scarce aerial hyphae and spore formation. (c) Scanning electron micrographs of S. coelicolor A3(2) strains M130, M130ΔamfC and M130 harbouring pKU209-amfC. Strains M130 and M130 harbouring pKU209-amfC form coils of spore chains. The micrograph of mutant M130ΔamfC is of a relatively abundantly sporulating area in the colony.
amfC mutations caused no detectable effects on streptomycin or A-factor production (Kudo et al., 1995).

(iv) The amfC mutation had no detectable effect on secondary metabolism in strain M145. The prototrophic strain M145 produces much more actinorhodin and undecylprodigiosin than strain M130. Comparison of actinorhodin production by strains M145 and M145∆amfC did not show any detectable difference (Fig. 1b). The two strains produced almost the same amount of blue pigments in R2YE liquid culture (data not shown), as well as on solid medium. The parental and M145∆amfC strains also produced undecylprodigiosin in the same time frame and in the same yield. As discussed below, we assume that strain M145 overproduces actinorhodin and undecylprodigiosin due to the sre-1 mutation (Ochi & Hosoya, 1998), and this mutation overcomes the defect in amfC. Since mutant M130∆amfC showed the same defects on media exogenously supplemented with histidine at 50 μg ml^{-1} and uracil at 7.5 μg ml^{-1}, the bisAI or uraAI mutation did not interfere with the defects in physiological and morphological development caused by disruption of amfC.

(v) Lack of spore pigment formation by S. coelicolor A3(2) harbouring extra copies of amfC. To investigate possible effects of overexpression, we introduced amfC on a high-copy-number plasmid, pIJ486 (pIJ486-amfC), into strains M130 and M145. The transformants grew normally and, on R2YE, R2, TSB and SFM solid media, their colonies became white (indicative of aerial hyphae formation) at the same time as the parental strains. The colonies also developed spores at the same time as the parental strains. However, they remained white even after 7 d, whereas colonies of the parents turned grey due to synthesis of the polyketide spore pigment (Davis & Chater, 1990). This effect of amfC on spore pigment formation was also observed with pKU209-amfC whose copy number is reported to be 1–2 per genome (Kakinuma et al., 1989) (Fig. 4). The white area in the colony of pIJ486-amfC- or pKU209-amfC-harbouring strains was full of spores with the same shape and size as those of the strains lacking the plasmids (Fig. 1c). In addition, coils of spore chains of strains M130 and M145 harbouring pKU209-amfC were indistinguishable from those of strains that did not harbour the plasmids. The spores with no pigment remained white after prolonged incubation. Thus, even one or two extra copies of amfC abolished spore pigment formation in strains M130 and M145. Since spore pigment formation by strains M130∆amfC and M145∆amfC harbouring pKU209-amfC was unstable, a very slight increase in the amount of AmfC seemed to suppress pigment formation.

Fig. 2. Actinorhodin production by S. coelicolor A3(2) strains M130 (○), M130∆amfC (●) and M130∆amfC harbouring pKU209-amfC (▲). Each plot is the mean of values obtained from three independent cultures in R2YE medium.

in place of glucose. The effect of the amfC disruption on actinorhodin production could also be detected on R2 and TSB media. On SFM medium, both the parental strain and M130∆amfC produced little actinorhodin. For better quantification (Fig. 2), we examined production of the blue pigment in R2YE liquid cultures by measuring the A540, reported to reflect mainly the amount of actinorhodin (Bystrykh et al., 1996). The amount of the pigment produced by M130∆amfC in liquid culture was also reduced. Introduction of pKU209-amfC restored pigment production to the same level as in the parental strain, both on solid and in liquid media. Thus, it was evident that amfC influenced actinorhodin production in strain M130.

We examined production of undecylprodigiosin, another pigmented antibiotic produced in the mycelium of S. coelicolor A3(2), by macroscopic observation of the lower surfaces of mycelial mats. This indicated that M130∆amfC produced undecylprodigiosin at a much later stage of growth than the parent. Measurements of undecylprodigiosin extracted from mycelium grown on solid medium, purified by TLC and analysed on a scanning spectrophotometer (Fig. 3) gave results consistent with the macroscopic observation. At 7 d after inoculation, when the amount produced by the parental strain reached a maximum, M130∆amfC produced almost no undecylprodigiosin. However, at 9 d after inoculation, M130∆amfC began to produce undecylprodigiosin, indicating that the amfC mutation delayed but did not prevent production. All of these observations suggested that amfC mutations in strain M130 influenced secondary metabolism as well as morphogenesis, in contrast to the situation in S. griseus where
amfC and development in S. coelicolor A3(2)

Transcription of amfC was examined by high-resolution S1 nuclease mapping with RNA prepared from mycelium grown on R2YE agar medium (Fig. 5a). The amfC mRNA was detected throughout growth, as was brdB mRNA encoding one of the major σ factors (Buttner et al., 1990; Shina et al., 1991); mRNAs were investigated in substrate mycelium obtained after 2 d growth and in a mixture of aerial mycelium and spores obtained after 6 d. The transcriptional start point was determined to be the G residue 29 nucleotides upstream of the ATG start codon. In front of the transcriptional start point, GTCACA and CACGAT with a 19 bp spacer are present (Fig. 5b). These sequences show similarity to those (TTGACA for −35 and TATAAT for −10 with a 17 bp spacer) of other prokaryotic promoters, as well as to one type (TTGACA for −35 and TAGGAT for −10 with a 18 bp spacer) of Streptomyces promoter believed to be active during vegetative growth (Hopwood et al., 1986).

Transcriptional analysis of actII-ORF4 in amfC disruptants

actII-ORF4 is a pathway-specific transcriptional activator for the whole cluster of actinorhodin biosynthetic genes (Fernández-Moreno et al., 1991; Fujii et al., 1996). Since the amfC-disrupted strains produced greatly reduced amounts of actinorhodin, we examined transcription of actII-ORF4 by S1 nuclease mapping with RNA prepared from mycelium grown on R2YE agar medium (Fig. 6a). In strain M130, actII-ORF4 mRNA with the expected size of 390 bp was detected after 2 d when cells grew as substrate mycelium, and it was still detected after 3 d when aerial hyphae appeared and after 4 d when cells grew as a mixture of aerial hyphae and spores. This is perhaps due to the mRNA preparation procedure; the harvested cell material used for RNA extraction was not fractionated and thus the late samples were mixtures of vegetative and aerial mycelium and spores. On the other hand, no actII-ORF4 mRNA was detected in M130amfC, although brdB mRNA was detected throughout growth. We concluded that the amfC disruption inhibited actII-ORF4 transcription, which resulted in severe reduction in actinorhodin yield.

Transcriptional analysis of whiE in strain M145 containing extra copies of amfC

The whiE gene cluster specifies the polyketide spore pigment in S. coelicolor A3(2), and two divergently oriented promoters, whiEP1 and whiEP2, are responsible for transcription of the gene cluster (Kelemen et al., 1998). The whiEP1 promoter transcribes most of the grey pigment production genes encoding ketsynthase,
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chain length factor, acyl carrier protein, aromatase and cyclase. Although both strains M130 and M145 containing extra copies of \( amfC \) did not form the spore pigment, we analysed transcription of \( whiE \) in strain M145 harbouring pKU209-\( amfC \) because M145 formed spores more abundantly than M130. We also examined \( whiE \) transcription in M145\( amfC \) for comparison. RNA was prepared from mycelium grown on SFM solid medium because M145 formed more spores on this medium than on any other medium tested. In the parental strain M145, the \( whiE \) mRNA with the expected size of 116 bp was detected in a large amount after 2 d of growth when aerial hyphae began to appear and decreased thereafter (Fig. 6b). Although the mRNA was still detected at 3 d when sporulation began, this is perhaps due to the mRNA preparation procedure, as described above. The \( whiE \) mRNA pattern in strain M145 was in agreement with that reported by Kelemen \textit{et al}. (1998). In strain M145 harbouring pKU209-\( amfC \), a small amount of the \( whiE \) mRNA appeared at 4 d. We assume that this mRNA derived from mycelium in the perimeter of the mycelium patch where the grey spore pigment was formed (Fig. 4). Thus, lack of pigment formation due to extra copies of \( amfC \) resulted from inhibition of \( whiE \) transcription.

In strain M145\( amfC \), the \( whiE \) mRNA was detected at 3 and 4 d. Strain M145\( amfC \) began to form aerial

Fig. 6. Transcription of \( \text{actII-ORF4} \) in \textit{S. coelicolor} A3(2) strains M130 and M130\( amfC \) and of \( \text{whiE} \) in \textit{S. coelicolor} A3(2) M145, M145\( amfC \) and M145 harbouring pKU209-\( amfC \). (a) RNA was prepared from cells grown on cellophane on the surface of R2YE agar at 30 °C for the time indicated (in days). Under these conditions, strain M130 began to form aerial mycelium (Am) at 3 d after inoculation, whereas mutant M130\( amfC \) began to form aerial hyphae at 4 d. In strain M130, \( \text{actII-ORF4} \) mRNA of 390 bp was produced just before aerial mycelium formation. In mutant M130\( amfC \), only a very faint signal at this position was seen. The \( 32\text{P} \)-labelled probe (lane P) was included as a control. \( hrdB \) mRNA was produced throughout growth in both strains M130 and M130\( amfC \). (b) RNA was prepared from cells grown on cellophane on the surface of SFM agar at 30 °C for the time indicated (in days). Under these conditions, strains M145 and M145 harbouring pKU209 formed aerial mycelium (Am) at 2 d after inoculation and spores (Sp) at 3 d, whereas mutant M145\( amfC \) formed less abundant aerial hyphae and spores at 3 and 4 d, respectively. In strains M145 and M145\( amfC \), \( \text{whiE} \) mRNA of 116 bp was produced at the onset of aerial mycelium formation. In strain M145 harbouring pKU209-\( amfC \), however, little \( \text{whiE} \) mRNA was produced. In all three strains, \( hrdB \) mRNA was produced throughout growth.

Fig. 5. Transcriptional analysis of \( amfC \) in \textit{S. coelicolor} A3(2) M130. (a) High-resolution S1 mapping of \( amfC \) mRNA prepared from cells grown on cellophane on the surface of R2YE medium at 30 °C for the time indicated (in days). S1-protected fragments were analysed in parallel with the sequence ladders (lanes G for the time indicated (in days). S1-protected fragments were analysed in parallel with the sequence ladders (lanes G + A and T + C). The \( 32\text{P} \)-labelled probe alone (lane P) was included as a control. The arrowhead indicates the position of the S1-protected fragment. The 5' terminus of the mRNA was assigned to the indicated position because the fragments generated by the chemical sequencing reactions migrate 1-5 nt further than the corresponding fragments generated by S1 nuclease digestion of the DNA–RNA hybrids (half a residue from the 3'-terminal phosphate group and one residue from the elimination of the 3'-terminal nucleotide) (Sollner-Webb & Reeder, 1979). The time course of \( hrdB \) mRNA abundance was determined using the same mRNA preparations. (b) Nucleotide sequence of the \( amfC \) promoter region. The transcriptional start point is shown by an arrow. Probable –35 (GTCACA) and –10 (CAGGAT) sequences, separated by a 19 nucleotide spacer, are underlined.
DISCUSSION

Disruption of \textit{amfC} in \textit{S. coelicolor} A3(2) severely reduced the formation of aerial mycelium, irrespective of the carbon source in the medium. Many \textit{bld} mutants (Merrick, 1976; Chater, 1989) show defects in aerial mycelium formation and antibiotic production, depending on the carbon source. \textit{amfC} disruptants of \textit{S. griseus} also showed sparse sporulation and \textit{amfC} from \textit{S. coelicolor} A3(2) restored its sporulation to normal (Kudo et al., 1995). Introducing \textit{amfC} from \textit{S. griseus} into \textit{S. coelicolor} A3(2) and \textit{S. lividans} abolished spore pigment formation (data not shown), consistent with the idea that \textit{amfC} in both strains is functionally the same. Since \textit{amfC} appears to be distributed widely among \textit{Streptomyces} spp. (Kudo et al., 1995), we assume that it plays a common, but not essential, regulatory role in aerial mycelium formation in this genus. Lack of spore pigment formation by several other \textit{Streptomyces} species harbouring \textit{P. J486-amfC} also supports this idea.

The \textit{amfC} mutants of \textit{S. coelicolor} A3(2) M130 produced much less actinorhodin than the parent. In addition, undecaprodigiosin production was delayed. The difference in the effect of \textit{amfC} mutations on these antibiotics may reflect differences in the regulatory cascades leading to transcription of the respective pathway-specific regulatory genes (Bibb, 1996). Because of the difference in regulation, the timing of production of these antibiotics is also different. The influence of \textit{amfC} on secondary metabolism in \textit{S. coelicolor} A3(2) is in contrast to the situation in \textit{S. griseus}, where \textit{amfC} mutations did not cause any detectable effect on \textit{A}-factor or streptomycin production (Kudo \textit{et al.}, 1995). In the absence of \textit{amfC}, almost no transcription of the pathway-specific transcriptional regulator \textit{actII}-ORF4 occurs, and thus actinorhodin biosynthetic genes are not transcribed. This means that \textit{amfC} is required for a particular signal transduction pathway to start transcription of the pathway-specific regulatory gene. In \textit{S. griseus}, the corresponding signal transduction pathway leading to streptomycin biosynthesis may not require the \textit{amfC} product. It is also possible that \textit{amfC} affects streptomycin production, but to an undetectable level.

\textit{amfC} mutations did not affect the yield of actinorhodin produced by \textit{S. coelicolor} A3(2) M145. Ochi & Hosoya (1998) identified a mutation, \textit{src-1} (suppression of \textit{relC} effect), to which they ascribed the overproduction of actinorhodin by strain M145. The \textit{src-1} mutation phenotypically suppresses or overcomes the defect in \textit{amfC} mutants that affects actinorhodin and undecaprodigiosin production by strain M145. The \textit{src-1} mutation, which is perhaps in the ribosomal protein S12 (Shima \textit{et al.}, 1996), exerts its effect at the translational level. Since \textit{amfC} exerts its influence on secondary metabolism at the translational level, \textit{src-1} seems to suppress the \textit{amfC} mutation in an independent way.

Extra copies of \textit{amfC} in \textit{S. coelicolor} A3(2) M130 and M145 caused no detectable effects on the morphology of aerial mycelium or spore chains, suggesting that the \textit{whi} genes (\textit{whiA}, \textit{B}, \textit{G}, \textit{H}, \textit{I}, and \textit{J}) required for sporulation septum formation in the regulatory cascade of aerial hyphae development function normally (Kelemen \textit{et al.}, 1998; Ryding \textit{et al.}, 1998). However, the absence of spore pigmentation in these strains due to decreased transcription of \textit{whiE} suggests that an increase in the amount of AmfC disturbs a regulatory signal to commence transcription of the \textit{whiE} promoters in the hierarchy of gene expression controlling aerial mycelium development and spore maturation. Because overexpression of \textit{amfC} phenotypically separates morphogenesis of spore chains from spore pigment formation, genetic studies using \textit{amfC} will elucidate the molecular basis for the steps of spore maturation.

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