Possible involvement of cAMP in aerial mycelium formation and secondary metabolism in Streptomyces griseus

Dae-Kyung Kang,1 Xin-Ming Li,1 Kozo Ochi2 and Sueharu Horinouchi1

Author for correspondence: Sueharu Horinouchi. Tel: +81 3 3812 2111 ext. 5123. Fax: +81 3 5802 2931. e-mail: asuhori@hongo.ecc.u-tokyo.ac.jp

1 Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
2 National Food Research Institute, Tsukuba, Ibaraki 305-8642, Japan

In Streptomyces griseus, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) triggers secondary metabolism and morphogenesis by binding a repressor protein (ArpA) and dissociating it from DNA. UV-mutagenesis of the A-factor-deficient mutant HH1 generated strain HO2, defective in the synthesis of ArpA and therefore able to form aerial mycelium, spores and streptomycin. Shotgun cloning of chromosomal DNA from wild-type S. griseus in strain HO2 yielded a gene that suppressed aerial mycelium formation and streptomycin production. Nucleotide sequencing and subcloning revealed that the gene encoded a eukaryotic-type adenylate cyclase (CyaA). In mutant HO2 production of cAMP was growth-dependent until the middle of the exponential growth stage; the production profile was the same as in the wild-type strain. However, the amount of cAMP produced was five times larger when mutant HO2 harbourcd cyaA on the high-copy-number plasmid pU486. Consistent with this, supplying cAMP exogenously at a high concentration to mutant HO2 suppressed formation of both aerial mycelium and streptomycin. On the other hand, some lower concentrations of cAMP stimulated or accelerated aerial mycelium formation. No effects of exogenous cAMP on morphogenesis and secondary metabolism were apparent in the wild-type strain. In addition, disruption of the chromosomal cyaA gene in the wild-type strain had almost no effect. Introducing cyaA cloned in either a low- or a high-copy-number plasmid suppressed morphogenesis and secondary metabolism not only in mutant HO2 but also in other arpA mutants, implying that the effects of cAMP became apparent in the arpA-defective background. When mutant HO2 carried cyaA on a plasmid, synthesis of the stringent response factor ppGpp was greatly reduced; this may account for the observed suppression by cAMP of morphogenesis and secondary metabolism. cAMP also affected protein tyrosine phosphorylation, as determined with anti-phosphotyrosine antibody.

Keywords: A-factor, A-factor receptor protein, protein tyrosine phosphorylation, stringent response

INTRODUCTION

Members of the bacterial genus Streptomyces produce a wide variety of secondary metabolites including antibiotics and other biologically active substances. Streptomyces spp. are also characterized by their complex morphological differentiation culminating in sporulation (Chater, 1993). We have studied a diffusible low-molecular-mass signalling molecule, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone), which triggers formation of both streptomycin and aerial mycelium in Streptomyces griseus (for reviews, see Khokhlov, 1982, 1988; Horinouchi & Beppu, 1990, 1992, 1994; Horinouchi, 1996). A-factor acts as a switch by binding to DNA-bound A-factor receptor protein (ArpA), dissociating it from the DNA, and releasing its...
repressor function (Onaka & Horinouchi, 1997; Onaka et al., 1997). The intracellular concentration of A-factor thus determines the timing of secondary metabolism and aerial mycelium formation. A series of S. griseus KM mutants and S. griseus HO1 of which are defective in ArpA, produce streptomycin and form aerial mycelium and spores even in the absence of A-factor (Miyake et al., 1989, Onaka et al., 1995). Introducing the intact arpA gene on a plasmid into these strains represses both streptomycin production and aerial mycelium formation. However, mutant HO2, which also has a defect in the biosynthesis of ArpA, is not repressed by introducing arpA on a plasmid. These observations prompted us to examine the characteristics of mutant HO2.

This paper describes properties of S. griseus mutant strain HO2, which has led us to notice the possible involvement of cAMP in secondary metabolism and cell differentiation in S. griseus. Ragun & Vining (1978) observed a possible, but indirect, relationship between cAMP and streptomycin biosynthesis by determining the timing of cAMP production in relation to that of streptomycin. We have confirmed their observations and further demonstrated distinct phenotypic changes dependent on cAMP by using mutant HO2. Very recently, Süssrunk et al. (1998) showed pleiotropic effects of cAMP on germination, secondary metabolism and cell differentiation in Streptomyces coelicolor A3(2). All of these observations suggest that cAMP exerts general pleiotropic effects on secondary metabolism and morphological development in Streptomyces spp.

METHODS

Bacterial strains, plasmids and culture media. S. griseus IFO 13350 was obtained from the Institute of Fermentation, Osaka. Streptomyces strains were grown in YMPD medium (yeast extract (Difco), 0.2 %; meat extract (Wako Pure Chemicals), 0.2 %; Bacto-peptone (Difco), 0.4 %; NaCl, 0.5 %; MgSO4·7H2O, 0.2 %; glucose, 1 %; and glycine, 0.5 %; pH 7-2). YMPD agar medium buffered with 25 mM TES (pH 7.2; Sigma) was used for examining phenotypes of S. griseus. Thiostrepton (15 µg ml⁻¹) and neomycin (20 µg ml⁻¹) were added when necessary. R2YE medium (Hopwood et al., 1985) containing 0.2 % asparagine instead of proline was used to regenerate protoplasts of S. griseus. Plasmids pJ486 and pJ487, both with a copy number of 40-100 (Ward et al., 1986), and pKU209, with a copy number of 1-2 (Kakinuma et al., 1991), were used as cloning vectors in S. griseus; they conferred thiostrepton resistance. Plasmid pARPLI containing arpA was previously described by Onaka et al. (1995). Plasmid pUC19 was used for DNA manipulation in Escherichia coli strains JM109 and JM110 (Yanisch-Perron et al., 1985). E. coli strains were routinely cultured in Luria broth (Sambrook et al., 1989) containing 0.2 % Bacto-peptone (Difco), 0.4 % meat extract (Wako Pure Chemicals), 0.5 % glucose, 1 % and ammonium sulfate was added to the supernatant to give a final concentration of 60 % (w/v). The precipitate was collected, dissolved in buffer A (50 mM triethanolamine and 0.5 M KCl, pH 7.0) and dialysed against buffer A. The assay mixture containing 200 µl of the crude lysate (5 mg protein) and 30 nM [3H]A-factor [2·8 Ci mmol⁻¹ (103·6 GBq mmol⁻¹)] was incubated at 30 °C for 30 min in the presence or absence of a 500-fold molar excess of nonlabelled A-factor and immediately applied to a Sephadex G-25 column (1·2 by 6 cm; Pharmacia PD10) for rapid separation of receptor-bound from free [3H]A-factor. Fractions 3 and 4 always contain ArpA-bound [3H]A-factor (Miyake et al., 1989; Onaka et al., 1995).

Shotgun cloning of cyA. Chromosomal DNA from S. griseus IFO 13350 was isolated and digested with BamHI; 2-20 kb fragments were purified by agarose gel electrophoresis. The fragments were ligated with BamHI-digested pJ486 and the reaction mixture was used to transform protoplasts of S. griseus HO2. After protoplast regeneration on the modified R2YE medium, transformants were selected by replica plating on YMPD agar containing thiostrepton. From more than 6000 transformants, one colony showing a bald (Bld) phenotype was picked for further study.

Gene disruption. The chromosomal cyA gene was disrupted by the method of Oh & Chater (1997). The BamHI ends of the originally cloned 3 kb fragment were changed into EcoRI sites by use of an 8-mer linker and cloned in the EcoRI site of pUC19. The 654 bp Aor51HI fragment in the cyA-coding sequence was replaced with a 1·32 kb Smal fragment containing a neomycin phosphotransferase (neo) gene from Tn5 (Beck et al., 1982) by standard DNA manipulation. The 3·6 kb EcoRI fragment containing the neo-disrupted cyA gene was purified by agarose gel electrophoresis, alkali-denaturation, and introduced by transformation into S. griseus strains IFO 13350 and HO2. After neomycin (20 µg ml⁻¹)-resistant transformants had been selected, true disruptants were chosen based on the results from Southern hybridization of BamHI-digested chromosomal DNA with the neo sequence and the 3 kb fragment on pHCA3 as probes. The probes were labelled with horseradish peroxidase by using an ECL kit (code RPN3000; Amersham).

Assay of streptomycin production in the presence of cAMP. Freshly prepared spores of S. griseus strains were used to inoculate 10 ml YMPD medium. cAMP at a final concentration of 0.25 or 2.5 mM was added at inoculation and at 9, 18, 23 and 34 h after inoculation. After 5 d cultivation, 200 µl of the culture broth on a paper disc was placed on agar medium seeded with Bacillus subtilis ATCC 6633. The yield of streptomycin was estimated from the diameter of the inhibition zone (Horinouchi et al., 1984).

Quantification of extracellular cAMP. S. griseus HO2 harbouring pJ486 or pHCA3 was grown at 28 °C for 3 d in YMPD medium containing 15 µg thiostrepton ml⁻¹. The mycelium was disrupted with a glass homogenizer and 1 ml was used to inoculate 100 ml of the same medium. Portions
Fig. 1. A-factor binding in S. griseus mutant HO2. (a) A-factor-binding activities in lysates prepared from the wild-type S. griseus IFO 13350 (top) and mutant HO2 (bottom) are shown. Fractions 3 and 4 represent ArpA-bound [3H]A-factor, and fractions 6–10 represent free [3H]A-factor. Specific binding is defined as the difference between binding of the radioactive A-factor in the presence (〇) and absence (●) of a 500-fold molar excess of nonlabelled A-factor. (b) Western blot analysis of the cell lysate with the anti-ArpA antibody. ArpA in the mycelium of S. griseus wild-type strain IFO 13350 and mutant HO2 grown for 36 h (corresponding to the mid-exponential phase) was detected by Western blotting with the anti-ArpA antibody. ArpA is detected in the mycelium prepared from wild-type strain IFO 13350 harbouring pI486 (–) or pCYA1 (+). Almost no ArpA is detected in the mycelium prepared from mutant HO2 harbouring either pI486 (–) or pCYA1 (+).

(5–10 ml) of the culture broth were taken out at intervals during cultivation at 28 °C, and supernatants were obtained by centrifugation at 5000 g for 5 min. The supernatants were boiled for 3 min and stored at –20 °C before use. The amounts of cAMP in the supernatants were measured with the Amersham enzyme immunoassay kit (code RPN225).

Quantification of intracellular ppGpp. Intracellular ppGpp was determined using HPLC apparatus equipped with a 25 cm Partisil PXS 10 SAX column (Whatman; void vol. 3.02 ml) according to the method of Ochi (1986). Spores (approx. 2 × 10⁸) were spread on a UV-sterilized cellophane sheet (diameter 8 cm) on the surface of YMPS agar medium. The cellophane sheet with mycelium was removed at intervals and quickly weighed. It was laid upside-down in 2 ml methanol in a Petri dish and 10 ml 1 M formic acid was added immediately. The entire procedure was completed within 10 s. After the sample had been extracted at 4 °C for 1 h, a supernatant was obtained by centrifugation and dried in vacuum; the amount of ppGpp present was measured by HPLC.

Preparation of cell extracts for Western blotting. S. griseus strains grown under similar conditions at 28 °C in YMPS medium were sampled (5–10 ml) at intervals and the mycelium was collected by centrifugation at 15000 g for 20 min. The mycelium was suspended in 2 ml 20 mM Tris/HCl (pH 7.0), 1 mM EDTA, 1 mM DTT and 10% (v/v) glycerol, and disrupted by sonication. The supernatants obtained by centrifugation of the sonicates at 5000 g for 10 min were used as crude extracts. When proteins with phosphotyrosines were detected, 100 μM Na₂VO₃ and 20 mM NaF were added to the sonication buffer to inhibit phosphatase activities. Protein concentrations were determined by the method of Bradford (1976) using BSA as a standard.

Western blot assay for detection of ArpA. Polyclonal antibody for ArpA was prepared in rabbits as described previously (Onaka & Horinouchi, 1997). For detection of ArpA in S. griseus strains, the crude extracts described above were electrophoresed in 0.1% SDS-10% polyacrylamide gels and electroblotted to a PVDF membrane (Immobilon-P; Millipore). The membrane was probed with the antibody and then incubated with horseradish-peroxidase-conjugated anti-rabbit IgG (Amersham). Signals were generated using the enhanced chemiluminescence system (ECL; Amersham).

Western blotting with anti-phosphotyrosine antibody. For immunological detection of phosphotyrosine-containing proteins, the crude extracts (20 μg protein) were electrophoresed in 0.1% SDS-10% polyacrylamide gels (7 × 9 cm). Western blotting was carried out as described by Matsudaira (1987) with the following modifications. The proteins were electro-transferred from the gel to a PVDF membrane at 200 mA for 2 h in CAPS buffer (pH 11.0). The blot was blocked overnight at 4 °C with 10% blocking reagent (Boehringer Mannheim) in TBS containing 10 mM Tris/HCl.
(pH 7.2) and 140 mM NaCl. The blot was then incubated with the peroxidase-conjugated anti-phosphotyrosine antibody (clone 3-365-10; Boehringer Mannheim), and diluted 1:1000 in TBS containing 5% blocking reagent for 2 h at room temperature. The blot was thoroughly washed with five changes of TBS containing 1% blocking reagent and developed with the BM chemiluminescent system (Boehringer Mannheim) according to the protocol of the manufacturer.

RESULTS

Properties of S. griseus mutant H02

S. griseus mutant H02 was derived as a sporulation-positive and streptomycin-producing mutant by UV-mutagenesis from the A-factor-deficient mutant strain HH1. Strain HH1 neither produces streptomycin nor forms aerial mycelium because of a deletion in afsA, which encodes a key enzyme for A-factor biosynthesis (Hara et al., 1983; Horinouchi et al., 1984; Ando et al., 1997a). The same strategy was previously used to isolate mutants defective in ArpA, such as a series of KM mutants (Miyake et al., 1990) and mutant HO1 (Onaka et al., 1997). Examination of an extract of mutant H02 showed very low, but detectable, A-factor-binding activity (Fig. 1a), suggesting that it contained a mutation that almost completely abolished either the A-factor-binding activity of ArpA or ArpA biosynthesis. Immuno-blotting with anti-ArpA antibody showed that mutant H02 produced a very small amount of ArpA (Fig. 1b). Although ArpA in mutant HO2 is slightly larger than in the wild-type strain, this tendency has been observed for all arpA-defective mutants so far examined, probably due to lack of processing at the carboxy-terminus. In wild-type S. griseus, ArpA seems to be processed at its carboxy-terminus (Onaka et al., 1995). A faint ArpA signal in mutant H02 was assumed to reflect the very low A-factor-binding activity. Mutant HO1, in which a mutated arpA encodes a protein with serine replacing proline-115 (Onaka et al., 1997), produced the altered ArpA protein from the early stage of growth. Mutants KM5, KM7 and KM12 also produced proteins reactive with the anti-ArpA antibody (data not shown), suggestive of a mutation in the arpA-coding sequence. It was therefore apparent that, in contrast to mutant HO1 and the KM mutants, mutant H02 had a defect in the biosynthesis of ArpA. Aerial mycelium formation and streptomycin production by these arpA mutants in the absence of A-factor was explained in terms of the repressor-type behaviour of ArpA (Miyake et al., 1990; Onaka et al., 1995, 1997). However, introduction of plasmid pARPH1 containing arpA on the high-copy-number vector pIJ487, or of pARPL1 containing arpA on the low-copy-number vector pKU209, into mutant H02 did not repress aerial mycelium formation or streptomycin production (data not shown). This result contrasts with that for other arpA mutants such as HO1 and KM7, where arpA on the plasmids almost completely represses both phenotypes (Onaka et al., 1995, 1997). Mutant H02 was thus assumed to contain a defect(s) that abolished both ArpA biosynthesis and the signal relays to ArpA to the downstream genes.

Cloning a gene that repressed sporulation of S. griseus HO2

To reveal the defect in mutant H02 we tried to clone a gene that repressed aerial mycelium formation and streptomycin production. By shotgun-cloning BamHI-digested chromosomal DNA from the wild-type S. griseus IFO 13350 into mutant H02 with a high-copy-number vector, pIJ486, we isolated a transformant that showed a Bld phenotype. Fig. 2(a) shows the Bld phenotype of mutant HO2 harbouring pHCYA1 (see below). This transformant produced a much smaller amount of streptomycin than did mutant HO2 harbouring the vector pIJ486 (Fig. 2b). Production of a diffusible yellow pigment, one of the secondary metabolites produced by S. griseus, was also repressed (data not shown). The cloned gene thus appeared to repress or inhibit both cellular differentiation and secondary metabolism in mutant HO2. However, as described below, introduction of pHCYA1 into the wild-type strain IFO 13350 did not cause any detectable phenotypic changes.

The recombinant plasmid in the Bld colony contained a 3 kb BamHI fragment in the BamHI site of pIJ486
CAMP and physiology in *S. griseus*

Fig. 4. CAMP production by *S. griseus* mutant HO2 harbouring pHCYA1 as a function of cultivation time. (a) Growth measured as wet cell weight. Mutant HO2 harbouring pJ486 (●), as a control, or pHCYA1 (○) was grown in YMPD liquid medium. (b) Amounts of CAMP in the culture broth determined by the immunological method. The peaks of CAMP biosynthesis at 40 and 48 h appear to coincide with the decision points of mutant HO2 harbouring pJ486 and pHCYA1, respectively.

Subcloning of *orf1*

A 1.6 kb *NruI–BamHI* fragment was excised from pHCYA3 and subcloned between the *HincII* and *BamHI* sites of pUC19. The fragment was then excised as an *EcoRI–HindIII* fragment and inserted between the *EcoRI* and *HindIII* sites of pJ486 to give pHCYA1. For construction of pLCYA1, the same *NruI–BamHI* 1.6 kb fragment was blunt-ended with Klenow fragment and 8-mer *EcoRI* linkers were attached to the ends. The *EcoRI* fragment thus generated was inserted in the *EcoRI* site of pKU209. For construction of pHCYA2, the 1.9 kb *SacI* fragment was first cloned into the *SacI* site of pUC19. The fragment was then excised as an *EcoRI–HindIII* fragment and inserted between the *EcoRI* and *HindIII* sites of pJ486. On the basis of these subcloning experiments (Fig. 3), we identified *orf1* in either the high-copy-number plasmid pJ486 (plasmid pHCYA1) or the low-copy-number plasmid pKU209 (plasmid pLCYA1) to be responsible for repression of aerial mycelium formation and streptomycin production in mutation HO2.

A computer-aided search with the CLUSTAL W program showed that *orf1* encoded a protein with end-to-end similarity in amino acid sequence to the adenylate cyclase from *S. coelicolor* A3(2) (Danchin et al., 1993). The carboxy-terminal portion of Orf1 also showed great similarity to the catalytic domains of adenylate cyclases from a prokaryote, *Brevibacterium liquefaciens* (Peters et al., 1991), and two eukaryotes, *Saccharomyces cerevisiae* (Kataoka et al., 1985) and *Schizosaccharomyces pombe* (Yamawaki-Kataoka et al., 1989). CyaAs of *S. griseus* and *S. coelicolor* A3(2) showed no sequence similarity to those from *E. coli* (Aiba et al., 1984), *Bacillus anthracis* (Escuyer et al., 1988) or *Erwinia chrysanthemi* (Danchin & Lenzen, 1988). Supporting the identification of *orf1* as an adenylate cyclase (*cyaA*) gene, the transformants harbouring *orf1* on a plasmid produced large amounts of CAMP, as described below. Thus, the *S. griseus* CyaA showing end-to-end similarity

(plasmid pHCYA3; Fig. 3). We purified the recombinant plasmid DNA from the transformant and constructed a restriction map of the insert (Fig. 3). The nucleotide sequence of the cloned fragment revealed the presence of two complete ORFs (Orf1 with 399 amino acids and Orf2 with 249 amino acids) and one truncated ORF (Orf3).

**Fig. 3.** Restriction map, sequence analyses and subcloning of the 3.0 kb *BamHI* fragment cloned on pJ486. The location and direction of ORFs were determined by analysis of the nucleotide sequence using the FRAME program (Bibb et al., 1984) with a sliding window of 100 codons. The subcloned fragments are on a high-copy-number plasmid, pJ486 (pHCYA series), and a low-copy-number plasmid, pKU209 (pLCYA1). The results of subcloning of the fragments are assessed from their ability to repress aerial mycelium formation by *S. griseus* mutant HO2.

<table>
<thead>
<tr>
<th>Repression</th>
<th>+</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHCYA1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHCYA2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pHCYA3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pLCYA1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CAMP production by *S. griseus* mutant HO2 harbouring pHCYA1 as a function of cultivation time.
Fig. 5. Acceleration (bottom) and inhibition (top) of aerial mycelium and spore formation by exogenously supplemented cAMP in *S. griseus* mutant HO2. Spore suspensions of mutant HO2 were spread on YMPD agar and paper discs containing 15 μmol or 5 μmol cAMP were placed on the agar surface at the indicated times after inoculation. The photographs were taken after 2 d. At 7 d after inoculation, sporulation occurred over all of the bottom Petri dish, and in the area outside the accelerated zone in the upper Petri dish. In the area close to the paper discs containing 15 μmol cAMP, where aerial mycelium formation was repressed, only substrate mycelium was observed, even at 10 d after inoculation.

to those of *S. coelicolor* A3(2) and *B. liquefaciens* belongs to a family of eukaryotic adenylate cyclases (see also discussion by Danchin *et al.*, 1993 and Peters *et al.*, 1991), rather than to the two types of prokaryotic enzymes.

Orf2 exhibits 77% identity in amino acid sequence to *B. subtilis* BirA, which regulates biotin synthesis (Bower *et al.*, 1995). The truncated Orf3 has strong sequence similarity to the β chain of the propionyl-CoA carboxylase (Donadio *et al.*, 1996), which participates in the catabolic pathway of odd-chain fatty acids, isoleucine, threonine, methionine and valine. These two ORFs seem, therefore, to be functionally unrelated to CyaA.

**Production of cAMP by mutant HO2 carrying cyaA on a plasmid**

We measured by enzyme-immunoassay the amount of cAMP produced by mutant HO2 harbouring pHCYA1 (Fig. 4). Culture broths accumulated up to 192 pmol ml⁻¹ in a growth-dependent manner until mid-exponential phase; the cAMP then rapidly disappeared, presumably due to cyclic phosphodiesterase activity (Terry & Springham, 1981). Mutant HO2 harbouring pIJ486 produced only 36 pmol cAMP ml⁻¹ in the same time frame. The timing of the cAMP peak in both strains coincided with the 'decision point' associated with the growth hesitation at mid-exponential phase (Neumann *et al.*, 1996; Holt *et al.*, 1992). Mutant HO2 harbouring plCYA1 produced 82–98 pmol cAMP ml⁻¹ with its peak at the decision point. The wild-type strain IFO 13350 produced cAMP in the same pattern and in almost the same amount as mutant HO2 harbouring pIJ486. Our attempts to measure cAMP accumulated within the mycelium failed to show a detectable amount.

**Effects of exogenously added cAMP on morphogenesis and secondary metabolism**

Contrary to our expectations, adding 5 μmol cAMP exogenously on a paper disc to *S. griseus* mutant HO2 at inoculation resulted in rapid aerial mycelium formation around the disc in 2 d as observed by microscopy (Fig. 5). cAMP presumably diffused rapidly into the agar, and by 4 d, aerial mycelium was present throughout the Petri dish. Accelerated aerial mycelium formation, and accordingly sporulation, was seen with cAMP in the range of 0·5–5 μmol per paper disc, and was more evident around the discs applied in the early stages of growth; when the discs were applied a long time after inoculation, the areas where aerial mycelium formed were smaller. These observations suggested that cAMP at a certain concentration caused precocious aerial mycelium formation. Because a decrease in pH due to acid accumulation in a *cyaA*-disruptant of *S. coelicolor* A3(2) disturbed antibiotic biosynthesis and morphological development (Susstrunk *et al.*, 1998), we examined the effect of buffering the medium. However, the same phenotypic changes caused by exogenous cAMP were observed on medium supplemented with 25 mM TES. No significant change of pH of the medium could be detected with a pH indicator strip. Dibutyryl cAMP, which is known to penetrate membranes readily (Posternak *et al.*, 1962), gave the same effects at the same concentrations as cAMP, suggesting that dibutyryl cAMP and cAMP are incorporated at similar rates in *Streptomyces*.

In amounts larger than 5 μmol per disc, cAMP had an inhibitory effect on aerial mycelium formation by mutant HO2 without affecting cell growth per se. Aerial mycelium formed in a doughnut-like area around paper discs containing 15 μmol cAMP applied at inoculation, indicating that stimulation of aerial mycelium formation was concentration-dependent. In 5 d, aerial mycelium and spore formation were observed in the area outside the doughnut zone, but even after 7 d little or no aerial mycelium formed inside the doughnut zone. This implies that cAMP at higher concentrations inhibits aerial mycelium formation. The inhibitory effect is more pronounced when the cAMP is supplied earlier, as judged by the larger diameters of inhibition zones. The same effects of cAMP were seen on the medium buffered with 25 mM TES.

When cAMP at a final concentration of 0·25 mM was added at inoculation and at 9, 18, 23 and 34 h after inoculation to cultures of mutant HO2 grown in YMPD medium, streptomycin titres were similar to those of
controls cultured in the absence of cAMP (data not shown). When 2.5 mM cAMP was added, however, almost no streptomycin was produced, irrespective of the timing of cAMP addition (data not shown). Thus although a low concentration of cAMP may not have stimulated streptomycin production to a detectable level, a high concentration clearly inhibited production.

Negligible effect of CAMP or overexpression of cyaA on the wild-type strain

When pHCYA1 was introduced by transformation into S. griseus strain IFO 13350, little change was detected in the timing and abundance of aerial mycelium formation, or in the timing and yield of streptomycin production (data not shown). Furthermore, exogenous supplementation from paper discs containing 5 and 15 μmol cAMP caused only minor apparent phenotypic consequences. By very careful comparison of aerial hyphae around paper discs containing 5 μmol and 15 μmol cAMP with hyphae from areas far from the discs, stimulatory and inhibitory effects, respectively, could be detected. This implies that CAMP does cause subtle effects on morphogenesis and secondary metabolism in the wild-type strain.

No detectable alteration of phenotypes in cyaA-disrupted strains

We constructed a cyaA null mutant from strain IFO 13350 by replacing the wild-type gene with a cyaA sequence disrupted by the neo gene sequence. We obtained three disruptants that gave the expected Southern hybridization pattern with two different probes (data not shown). These cyaA mutants produced no detectable amount of cAMP when assayed by the enzyme-immunological method (data not shown), indicating that strain IFO 13350 contains a single cyaA gene. However, the growth profile showing a decision point, the timing and abundance of aerial mycelium formation, and the timing and yield of streptomycin production by the disruptants were the same as those of the wild-type strain.

We also derived cyaA-disruptants from mutant HO2. Morphological development and secondary metabolism in these disruptants were not detectably different from those of mutant HO2. Exogenously supplied cAMP exerted the same effects on these disruptants as on mutant HO2 (data not shown). We also constructed cyaA-disruptants from the A-factor-deficient mutagen strain HH1, but these mutants showed only a Bld and streptomycin-negative phenotype, just like strain HH1.

Because Susstrunk et al. (1998) observed a much lower frequency of germination in cyaA-disrupted S. coelicolor A3(2) spores, we examined the germination of spores from cyaA-disruptants of S. griseus IFO 13350. When freshly prepared spore suspensions from the wild-type strain and one of the cyaA-disruptants were spread on YMPD agar and the numbers of colonies appearing at intervals were compared, no difference was observed. Counting the number of the germinated spores under a microscope also showed no measurable difference; both germinated with almost 100% frequency. Thus, disruption of cyaA did not appreciably affect germination of the S. griseus spores.

Change of the intracellular ppGpp pool in mutant HO2

Morphological and physiological differentiation is often a response to nutrient limitation. One of the bacterial regulatory systems coupled to nutrient limitation is the stringent response, which amongst other cellular reactions causes cessation of RNA synthesis (Cashel, 1975; Gallant, 1979). ppGpp is one of the compounds responsible for the stringent response. Its occurrence in S. griseus and possible relationship to streptomycin biosynthesis were studied by An & Vining (1978). Ochi (1987a) proposed that its intracellular accumulation triggers streptomycin biosynthesis and that a decrease in the GTP pool leads to aerial mycelium formation. To explore these possibilities we measured changes in the amount of ppGpp in mutant HO2 containing cyaA on a plasmid.

In mutant HO2 containing the vector plJ486, ppGpp synthesis on YMPD medium began with the onset of aerial hyphae formation and continued until the onset of sporulation (Fig. 6). The time course of ppGpp production relative to the developmental stages matched that in wild-type strain IFO 13350 (data not shown) and in S. griseus IFO 13189 (Ochi, 1987a), although morphological development in mutant HO2 started slightly later than in the wild-type strain. Mutant HO2 harbouring pHCYA1 accumulated a severely reduced amount of ppGpp. This could mean that the failure of mutant HO2 to produce streptomycin in the presence of pHCYA1 and probably in the presence of a large amount of exogenously added cAMP is caused by insufficient accumulation of ppGpp.

Phosphotyrosine proteins in mutant HO2

Waters et al. (1994) detected protein tyrosine kinases in various Streptomyces species and observed changes in the phosphorylation pattern depending on the growth stage and culture conditions. We also observed that protein serine/threonine and tyrosine kinase inhibitors such as staurosporine and K-252a inhibit aerial mycelium formation and antibiotic production in S. coelicolor A3(2) (Hong & Horinouchi, 1998) and in S. griseus (Hong et al., 1993). Attempts to examine protein phosphorylation patterns in mutant HO2 with and without pHCYA1 by in vitro phosphorylation with [γ-32P]ATP and cell lysates (Hong et al., 1993) failed because the phosphorylation patterns were not reproducible. Although phosphorylation of several proteins did appear to be affected by the presence of pHCYA1, we abandoned this method in favour of using anti-phosphotyrosine antibody to detect differences in protein phosphorylation on tyrosine residues. This procedure directly detects proteins with phosphotyrosine residues in the cell lysate. In S. griseus, changes in the
tyrosine phosphorylation pattern depended on the growth phase (Fig. 7), as has been reported in other Streptomyces species (Waters et al., 1994). In addition, as described below, phosphorylation patterns of the wild-type strain and mutant HO2 were totally different. This prompted us to examine protein phosphorylation in detail with the anti-phosphotyrosine antibody. In the wild-type S. griseus strain, Western blotting detected more than nine proteins (bands 1–9) in the wild-type S. griseus strain, all of which were clearly visible before the decision point. However, some of them (bands 4, 5, 6, 7 and 9) rapidly disappeared after that point, indicating that phosphorylation of these proteins was strictly under decision-point control.

In mutant HO2, on the other hand, phosphorylation of tyrosine residues in most of these proteins was severely repressed before the decision point (Fig. 8). Only proteins 1 and 3 were visible. At the decision point and thereafter, the phosphorylation of most of the proteins was apparent. In addition, two new phosphorylated proteins, x and y, appeared. Since the same phosphorylation patterns were observed in other arpA mutants (e.g. a series of KM mutants; data not shown), the absence of tyrosine phosphorylation on these proteins appeared to be due to the mutation in arpA. This contrasts sharply with phosphorylation of these proteins before the decision point and their subsequent dephosphorylation in the wild-type strain (Fig. 7). In mutant HO2 harbouring pHCYA1, phosphorylation of most proteins detected in the wild-type strain was repressed until the end of exponential growth. Because supplying cAMP exogenously to mutant HO2 at a final concentration of 10 mM also prevented phosphorylation, repression of phosphorylation throughout the exponential growth phase is attributed, directly or indirectly, to overproduction of cAMP. Although little is known about the relationship of proteins with phosphotyrosines to morphogenesis and

---

**Fig. 6.** Changes in the intracellular pool of ppGpp in S. griseus HO2. Mycelium of mutant HO2 harbouring pJ486 or pHCYA1 was spread on YMPD agar medium and incubated at 28.5 °C. (a) Growth observed visually. (b) ppGpp analysis by the intracellular ppGpp assay described in Methods of whole cells from one Petri dish. ppGpp accumulation begins concomitantly with aerial hyphae formation in mutant HO2 harbouring pJ486. Aerial mycelium (AM) and spores (S) were formed by mutant HO2 harbouring pJ486 at the points indicated. The amount of ppGpp accumulated in mutant HO2 harbouring pHCYA1 is much smaller than in mutant HO2 harbouring pJ486.

**Fig. 7.** Changes in the profile of proteins with phosphotyrosine residues in S. griseus IFO 13350. The wild-type strain IFO 13350 was grown in YMPD medium and mycelium was taken at the indicated times (points A–F) to detect proteins with phosphotyrosine residues by Western blotting with anti-phosphotyrosine antibody. Growth was measured as wet cell weight. The decision point apparently corresponds to point C. Nine reactive phosphorylated proteins (1–9) are clearly visible before the decision point.
CAMP and physiology in *S. griseus*... secondary metabolism, it is apparent that in several of them CAMP affects phosphotyrosine phosphorylation.

**DISCUSSION**

Because only very low A-factor-binding activity and almost no ArpA were detected in *S. griseus* mutant HO2, the mutation(s) in this strain seems to prevent ArpA production. The additional evidence that introduction of an intact *arpA* gene does not repress aerial mycelium formation or streptomycin production suggests that a signal relay from ArpA to downstream genes may also have been interrupted. Our attempt to identify the latter mutation in strain HO2 led to the cloning of *cyaA* encoding a eukaryotic-type adenylate cyclase. The stimulatory effect of a low concentration of CAMP on aerial mycelium formation and possibly also on secondary metabolism, and the inhibitory effect at higher concentrations, were observed not only in mutant HO2 but also in a series of KM mutants and in mutant HO1, all of which contain a mutation in *arpA*. Although CAMP provided either as an exogenous supply or by disrupting *cyaA* did not have a very distinct effect in the wild-type strain, it could be involved in regulating morphogenesis and secondary metabolism, though not essential to the processes. It might play a role in tuning the physiological conditions in a still unknown way, as is observed in prokaryotes (Botsford & Harman, 1992) and eukaryotes (Hunter, 1995; Taylor et al., 1990). The regulatory roles of *cyaA* in *S. coelicolor* A3(2) reported recently by Süssbrun et al. (1998) suggest that CAMP does act as a second messenger in *Streptomyces* spp. Tata & Menawat (1994) also reported a role for CAMP in tylosin synthesis in *Streptomyces fradiae*.

In *S. griseus*, A-factor is effective only during the short period when the decision to biosynthesize streptomycin is established and subsequent production of the antibiotic and differentiation can be influenced (Neumann et al., 1996). Consistent with this, supplying A-factor to A-factor-deficient mutant strains after the decision point induces little or no aerial mycelium formation (Ando et al., 1997b), and providing a large amount at inoculation severely disturbs aerial mycelium formation, probably due to too early an onset of the sequence of metabolic events (Ando et al., 1997b). A-factor is thus thought to initiate an ordered sequence of metabolic events required for morphological and physiological differentiation during the decision phase. It is noteworthy that the *arpA* mutations in mutants KM7 and HO1 cause the hosts to begin aerial mycelium formation and streptomycin production earlier than in the wild-type strain (Miyake...
et al., 1990; Onaka et al., 1997). We presume that the absence of ArpA sets off an earlier sequence of some particular metabolic events that alter the physiological conditions influencing other metabolic events, but lead finally to morphological and physiological differentiation. The profile of cAMP accumulation, which shows a peak exactly at the decision point, suggests a role for this molecule during the decision phase, as was previously pointed out by Ragan & Vining (1978). The effects of cAMP may be concealed in the wild-type background, and may become apparent only by their modulation, directly or indirectly, of the aberrant sequence of events induced by the absence of ArpA.

One of the metabolic events, actually affected independently of A-factor by cAMP, is synthesis of the stringent response factor ppGpp. This event not only reflects nutrient conditions, such as nitrogen limitation and amino acid starvation, but is important for initiating both physiological and morphological differentiation (Ochi, 1986, 1987a, 1988; Chakraburtty & Bibb, 1997). S. griseus mutant HO2, like the wild-type strain, began to accumulate ppGpp at the onset of aerial mycelial formation. In S. griseus IFO 13189, ppGpp was shown to be a strong inhibitor of IMP dehydrogenase, which eventually led to a decrease in the intracellular GTP pool size (Ochi, 1987b). We therefore assume that the inability to form aerial mycelium of mutant HO2 carrying a high-copy number of cyaA is due to a slower decrease in its GTP pool size. The greatly reduced accumulation of ppGpp when cyaA was introduced on the high-copy-number plasmid accounts for the observed repression of aerial mycelium and streptomycin production in these transformants. S. griseus mutant HO2 will offer a feasible system to examine the possible effect of cAMP on the expression of relA encoding ppGpp synthetase (Martinez-Costa et al., 1996; Chakraburtty & Bibb, 1997). Such an effect may not be readily detected in the wild-type strain, in which sporulates and produces streptomycin almost normally in the presence of a high concentration of cAMP.

We have recently found that an AfsK/AfsR homologous system controls aerial mycelium formation in response to glucose in S. griseus (T. UmeYame, P.-C. Lee, K. Ueda & S. Horinouchi, unpublished results). In S. coelicolor A3(2), a protein serine/threonine kinase, AfsK, and its target protein AfsR control secondary metabolism (Matsumoto et al., 1994). In the present study, we found that profiles of protein tyrosine phosphorylation changed dramatically after the decision point (Fig. 7). Similar changes in protein tyrosine phosphorylation have been reported in Streptomyces hygroscopicus, Streptomyces lividans and Streptomyces lavendulae (Waters et al., 1994). In mutant HO2, the tyrosine phosphorylation pattern is almost the reverse of that in the wild-type strain; most proteins phosphorylated before or at the decision point in the wild-type strain are not phosphorylated in mutant HO2 until after the decision point. Although the role of tyrosine phosphorylation in physiological and morphological differentiation is not yet clear, this finding suggests that mutant HO2 differentiates physiologically and morphologically in an aberrant protein tyrosine phosphorylation background. Since the tyrosine phosphorylation pattern in the A-factor-deficient mutant strain HH1 containing the intact arpA gene is similar to that in the wild-type strain (data not shown), the change in the phosphorylation pattern is ascribed to ArpA. Higher concentrations of cAMP either directly or indirectly inhibit tyrosine phosphorylation after the decision point in mutant HO2, and this may result in repression of physiological and morphological differentiation. Further studies are necessary to fully elucidate the role of tyrosine phosphorylation and to relate it to cAMP. Modulatory activities of cAMP are observed exclusively with protein serine/threonine kinases in eukaryotes (Taylor et al., 1990; Hunter, 1995), but activation of a protein tyrosine kinase in Acinetobacter calcoaceticus belonging to the Neisseriaceae family has been reported (Grangeasse et al., 1997).

In conclusion, our data, together with those of Susstrunk et al. (1998) and Tata & Menawat (1994), show a pleiotropic regulatory role for cAMP in Streptomyces. One effect is on ppGpp synthesis, reflecting the nutrient conditions closely associated with morphological and physiological differentiation. Protein tyrosine phosphorylation, also suggested to be important for differentiation in Streptomyces (Hong et al., 1993), is also affected by cAMP in conjunction with ArpA, one of the important proteins in the A-factor regulatory cascade. These findings suggest that a complex cAMP regulatory network participates in a coordinated and concerted way with other regulatory networks to control physiological functions.

ACKNOWLEDGEMENTS

This work was supported, in part, by the Nissan Science Foundation, by the Research for the Future Program of the Japan Society for Promotion of Sciences (JSPS), and by the Bio Design Program of the Ministry of Agriculture, Forestry, and Fisheries of Japan (BDP-99-VI-2-1).

REFERENCES


Received 20 November 1998; revised 13 January 1999; accepted 20 January 1999.