POSSIBLE INVOLVEMENT OF A MONOPHENOL MONOOXYGENASE HOMOLOGUE IN A-FACTOR-DEPENDENT YELLOW PIGMENT PRODUCTION IN *Streptomyces griseus*

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A-factor (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) is an essential hormonal regulator for morphological development, as well as streptomycin and yellow pigment productions in *Streptomyces griseus*. By UV-treatment, three mutants that were deficient in yellow pigment production but showed normal morphological development and streptomycin production were obtained. A 7.5 kb-DNA fragment, which restored the yellow pigment production in one of the mutants, was shotgun-cloned from a chromosomal DNA of wild-type *S. griseus*. Sequence analysis of this fragment showed that it contained 6 complete open reading frames including *melC1* and *melC2*. MelC2 and MelC1 have high sequence similarity to monophenol monooxygenases and co-factors of the enzyme, respectively. Because a *melC2*-disrupted strain showed yellow pigment deficient phenotype, it was probable that the monophenol monooxygenase homologue is involved in the biosynthesis of the yellow pigment.

Keywords: *Streptomyces griseus*, A-factor, yellow pigment, monophenol monooxygenase, secondary metabolism

INTRODUCTION

The filamentous, soil living, gram positive bacterial genus *Streptomyces* is characterized by the ability to produce more than 6,000 antibiotics and many physiologically active compounds. Most of these compounds are secondary metabolites and the production of secondary metabolites is genetically regulated. In *Streptomyces griseus*, which is a representative strain of *Streptomyces* because of streptomycin production, a small diffusible substance called A-factor was isolated as an autoregulatory signal molecule responsible for aerial mycelium formation, streptomycin (Sm) and yellow pigment (YP) productions [1, 2, 3]. A-factor deficient mutants can neither produce both Sm and YP nor form aerial mycelium or spores. However, exogenous supplementation of A-factor at a concentration as low as 10^{-9} M to these mutants restores aerial mycelium formation, sporulation, and Sm and YP production [1, 2, 3].

Recently, a major regulatory cascade leading to Sm production was revealed[4]. A-factor is gradually accumulated in a growth dependent manner by the action of AfsA probably condensing a glycerol derivative and a β–keto acid. When the concentration of A-factor reaches a certain critical level, it binds an A-factor specific receptor ArpA, which has bound and repressed the promoter of *adpA*, and dissociates ArpA from the promoter, thus leading to transcription and translation of a transcription factor AdpA. AdpA then activates transcription of *strR* by binding an upstream activation sequence. A pathway specific transcriptional activator StrR induced in this way activates transcription of most of Sm biosynthesis genes by binding multiple sites in the gene cluster.

Disruption of the chromosomal *adpA* resulted in not only complete inhibition of Sm production but also complete inhibition of aerial mycelium formation and YP production [4, 5]. This indicated that AdpA should activate transcription of some genes involved in aerial mycelium formation and YP production, in addition to *strR*. Very recently we identified *adsA* encoding an extracytoplasmic function σ factor as a target gene of AdpA [5]. Disruption of the chromosomal *adsA* gene resulted in loss of aerial hyphae formation but not Sm and YP production, indicating that σ^{adsA} is involved only in morphological development and not in secondary metabolic function [5]. On the other hand, little has been known about YP biosynthesis and its regulation. This is mainly because YP production is difficult to be observed on normal rich media such as YMPD. Recently, YP production was easily observed on phosphate-depleted minimal medium. This enabled us to do various kinds of experiments about YP production. The final goal of this study is to reveal both the YP biosynthetic pathway and its regulatory cascade, in which AdpA should play an important role as an A-factor-dependent transcriptional activator. Determination of the chemical structure of the yellow pigment is also in progress. This report would be describe isolation of the YP-deficient mutants, cloning of a DNA fragment that restored YP production in one of the mutants and sequence analysis of the cloned fragment. *melC2* encoding a monophenol monooxygenase homologue was identified as a probable YP-biosynthetic gene.

MATERIALS AND METHODS

Bacterial strains, plasmids and media

The wild type strain *S. griseus* IFO 13350 and an A-factor-deficient strain HH1 were described previously [6].

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S. griseus Δ*adpA* was also described previously [4]. High-copy-number plasmid pIJ702 carrying a thiostrepton resistance gene used as a cloning vector for *Streptomyces* [7] was obtained from D. A. Hopwood, John Innes Centre, Norwich,UK. *Escherichia coli* JM109 and pUC18 [8] for DNA manipulation were purchased from Takara Shuzo. Media for *S. griseus* such as YMPD [4], R2YE [7] and Bennett [6] were as described earlier. The phosphate-depleted minimal medium for YP production assay contains glucose (0.9% w/w), L-asparagine (0.9% w/w), $(NH_4)_2SO_4$ (0.2% w/w), NaCl (0.1% w/w), K_2SO_4 (0.05% w/w), $MgSO_4 \cdot 7H_2O$ (0.02% w/w), $CaCl_2 \cdot 2H_2O$ (0.01% w/w), Tris•HCl (0.242% w/w, pH 7.2), the trace element solution [7] (1%) and agar (2%). Media and growth conditions for *E. coli* were described by Maniatis et al [9]. Ampicillin at final concentration of 50 μg/ml was used for *E. coli* when necessary. For *S. griseus*, thiostrepton and neomycin were added at final concentrations of 50 and 15 μg/ml, respectively, when necessary.

General recombinant DNA techniques

Restriction endonucleases, T4 DNA ligase and other DNA-modifying enzymes were purchased from Takara Shuzo. Thiostrepton was a gift from Asahi Chemical Industry, Shizuoka, Japan. DNA was manipulated in *Streptomyces* [7] and *E. coli* [9], as described earlier. Nucleotide sequences were determined by the dideoxy chain termination method with the Thermo Sequenase fluorescence-labeled primer cycle sequencing kit (Amersham) on an automated DNA sequencer (Shimadzu, DSQ-2000L).

Isolation of the yellow pigment-deficient mutants of *S. griseus*

S. griseus spores 10 μl (mixture of spore solution 10 μl and 10 % glycerol 990 μl) suspended in 10 ml of 10% glycerol were mutagenized by UV irradiation (50 seconds) to give about a 0.5 % survival ratio. Mutagenized spores were then spread on YMPD agar medium and incubated 5-7 days at 28°C. About 5,300 sporulated colonies were picked up and transferred onto phosphate-depleted minimal medium and incubated for 3-4 days at 28°C for YP production assay. As the results, three YP-deficient mutants (M14, M28 and M31) were obtained.

Shotgun-cloning of a DNA fragment which restore YP-deficient phenotype of *S. griseus* M31

Chromosomal DNA from *S. griseus* IFO 13350 was digested partially with *Sau*3AI and 6 to 10-kb fragments were purified by agarose gel electrophoresis. The fragments were ligated with *Bgl*II-digested pIJ702 and the reaction mixture was used to transform protoplasts of a YP-deficient mutant strain M31. After protoplast regeneration on R2YE medium, about 6,000 transformants were transferred onto YMPD agar containing 50 μg/ml of thiostrepton and incubated for 5-7 days at 28°C. Then the transformants were inoculated on the phosphate-depleted minimal medium plate and incubated for 3-4 days at 28°C for YP production assay. From 2,600 transformants, two colonies producing the yellow pigment were picked for further study. The plasmids contained in these transformants were named pAYP10 and pAYP20, which carried an about 12-kb and a 7.5-kb *Sau*3AI fragment in the *Bgl*II site of pIJ702, respectively.

Nucleotide sequencing of the 7.5-kb fragment on pAYP20

pAYP20 was digested by *Eco*RV and *Bgl*II and then a 9.2-kb *Eco*RV-*Bgl*II fragment containing a part of pIJ702 sequence (1.7 kb) and whole cloned fragment (7.5 kb) was cloned into *Sma*I plus *Bam*HI digested pUC18, resulting in pAYP200. By using several kinds of restriction enzymes including *Sph*I, *Bam*HI, *Sac*I, *Sma*I, *Aor*51HI, *Pma*CI and *Nae*I, the insertion fragment of pAYP200 was divided into small fragments and they were cloned into pUC18. Nucleotide sequence of these subclones was determined. Open reading frames were predicted by the Frame Plot analysis of the nucleotide sequence [10].

RESULTS AND DISCUSSION

Isolation and characterization of the yellow pigment-deficient mutants of *S. griseus*

To clone a gene involved in YP production, the first is carried out isolation of some YP-deficient mutants by UV-treatment. About 5,300 mutagenized colonies were examined for YP production on phosphate-depleted minimal medium and three YP-deficient mutants (M14, M28 and M31) were obtained. All these three mutants were deficient only in YP production but not in Sm production and morphological development (Table 1). This indicated that the mutation points should be downstream from AdpA in the regulatory cascade, because disruption of *adpA* resulted in complete loss of aerial mycelium formation and Sm production as well as YP production. Therefore these mutants were available as a host strain for a shotgun-cloning experiment as described below.

Table 1. Phenotypes of the mutants and *mel*C2-disrupted strain

Strain of *S.griseus*	Morphological differentiation	Streptomycin production	Yellow pigment production
Wild- type	+	+	+
HH1	–	–	–
ΔadpA	–	–	–
adsA- km^r	–	+	+
M14	+	+	–
M28	+	+	–
M31	+	+	–
melC2-km^r	+	+	–

Cloning of the genes involved in YP production

Shotgun cloning method was used to obtain a DNA fragment that restored YP production in the YP-deficient strain M31 using genomic DNA library of wild-type *S. griseus*. As described in materials and methods, about 2,600 transformants were examined for YP production on phosphate-depleted minimal medium and two transformants producing the yellow pigment were obtained. The plasmids (pAYP10 and pAYP20) contained in these transformants were extracted and introduced again into protoplasts of the YP-deficient strain M31. This retransformation experiment confirmed that each plasmid restored YP production in the mutant. Furthermore, when each plasmid was introduced into *Streptomyces lividans,* it caused YP production, suggesting that these plasmids might contain YP biosynthetic genes themselves rather than the regulatory genes for YP biosynthesis.

Agarose gel electrophoresis of the DNA fragments resulted from restriction enzyme treatment of each plasmid revealed that pAYP10 and pAYP20 carried an about 12-kb and a 7.5-kb *Sau*3AI fragments in the *Bgl*II site of pIJ702, respectively. Furthermore, completely same size of fragments (for examples, 0.7-kb *Sph*I fragment, 1.6-kb *Sph*I fragment and 1.3-kb *Bam*HI fragment) were detected when each plasmid was digested by the restriction enzymes, indicating that the insert fragments of both plasmids should overlap. On this overlapping region (2.7-kb *Bam*HI-*Sph*I), *melC1* and *melC2* were encoded (see Fig. 1).

Nucleotide sequencing of the 7.5-kb fragment on pAYP20

Nucleotide sequence of the 7.5-kb fragment on pAYP20 was determined and translated into a computer-aided homology search (Fig.1). A computer-aided homology search revealed that more than half of the cloned fragment (from nucleotide (nt.) 1 to nt. 4331) was identical to a DNA fragment which had already been cloned and sequenced by K. Ueda *et al.*. They cloned this DNA fragment by a hybridization method using *melC* gene of *Streptomyces antibioticus* [11] as a probe (unpublished data).

Frame Plot analysis indicated that it contained 6 complete open reading frames (ORFs) and two truncated ORFs (Fig.1).Orf2 and Orf3 had low but significant sequence similarity to a coenzyme A ligase of *E. coli* (26% identity) and a putative aldehyde dehydrogenase of *Streptomyces coelicolor* A3(2) (27% identity), respectively. MelC2 had high sequence similarity (41-49% identity) to monophenol monooxygenases of various *Streptomyces* including *S. antibioticus*, *S. coelicolor* A3(2), *Streptomyces glaucescens, Streptomyces galbus*, *Streptomyces lincolnensis*, and *Streptomyces lavendulae*. MelC1 was also homologous to co-factors of the monophenol monooxygenases of those strains (36-41% identity). The co-factor was reported to be essential for the monophenol monooxygenase activity [12]. Orf4 had sequence similarity (36% identity) to a benzoate transport protein of *Pseudomonas putida*. Probably *melC1*, *melC2* and *orf4* were co-transcribed in a same transcriptional unit. Orf5, Orf6 and Orf7 had sequence similarity to a conserved hypothetical protein of *Methanococcus jannaschii* (42% identity), a conserved protein (putative aldolase) of *Methanobacterium thermoautotrophicum* (43% identity) and an aspartate kinase alpha chain of *Mycobacterium smegmatis* (31% identity), respectively.

As described below, *melC2* was probably involved in YP biosynthesis. Further subcloning analysis on the cloned fragment and gene disruption experiments would revealed possible involvement of the other gene products in YP production. Determination of the mutation points in the chromosomal DNA of the YP-deficient mutants would be required.

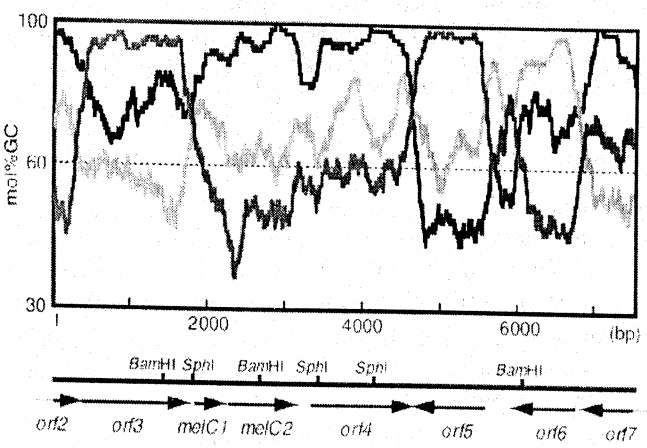

Figure 1. **Gene organization of the cloned DNA fragment**. Arrows indicate the extents and directions of ORFs predicted by Frame Plot analysis of the nucleotide sequence. Restriction map of the cloned fragment is also shown.

A *melC2*-disrupted strain showed YP-deficient phenotype

The *melC2*–disrupted strain was obtained and examined YP production on phosphate-depleted minimal medium. The *melC2*–disrupted strain was completely deficient in YP production (Table 1). This result indicated that MelC2 was an important enzyme for YP production.

The monophenol monooxygenases (tyrosinase) encoded by *melC2* of *S. antibioticus* was a key enzyme for melanin production in the strain [11]. *S. griseus* also produced brown melanin pigment, especially on a medium containing Cu^{++}. But the *melC2*–disrupted strain produced the melanin pigment, indicating that *melC2* should not be involved in the melanin production.

For further analyses on a functional role of MelC2 for YP production, determination of the chemical structure of the yellow pigment was absolutely required. It was also important to analyze the regulation mechanism of *melC2* expression for elucidation of the A-factor regulatory cascade leading to YP production.

CONCLUSION

1. Three mutants of *S.griseus* that showed normal morphological development and normal streptomycin production but were deficient in yellow pigment production were obtained by UV treatment.
2. Two plasmids (pAYP10 and pAYP20) that restored yellow pigment production in the mutant M31 were obtained by shotgun cloning.
3. The complete nucleotide sequence of the 7.5 kb fragment on pAYP20 was determined.
4. Frame analysis showed that the fragment contained 8 open reading frames including MelC1 and MelC2. MelC2 and MelC1 show high sequence similarity to monophenol monooxygenases and their co-factor proteins of *Streptomyces*, respectively.
5. A MelC2-disrupted strain showed yellow pigment-deficient phenotype indicating a possible involvement of melC2 in the biosynthesis of the yellow pigment in *S.griseus*.

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