# Characterization of Mutants Defective in Melanogenesis and a Gene for Tyrosinase of *Streptomyces griseus*

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Nitrosoguanidine-induced melanin-negative mutants of *Streptomyces griseus* were classified according to their ability to produce streptomycin and A-factor, and to form aerial mycelium. A large proportion of the mutants showed deficiency in either antibiotic production and morphological development or both, suggesting close regulatory correlation between melanogenesis and morphological and physiological differentiation. The tyrosinase-encoding *mel* operon of *S. griseus* was cloned and examined for its role in melanogenesis of this organism. As in other *Streptomyces* homologues, the operon consisted of two open reading frames, *melC1* encoding the putative cofactor and *melC2* encoding the tyrosinase. Regardless of the distinct sequence similarity, introduction of the operon on plasmids failed to confer melanin production in the melanin-negative mutants, and the disruption of *melC2* barely affected the melanin productivity, which indicated the presence of another enzyme involved in the melanogenesis in *S. griseus*. On the other hand, *mel* on a high-copy-number plasmid caused precocious aerial mycelium formation in *Streptomyces lividans* TK21 suggesting a stimulatory role of tyrosinase in morphological development.

The ability to produce melanin is a characteristic property of a number of *Streptomyces* species[1]. Melanin formation from L-tyrosine is mediated by tyrosinase (monophenol, dihydroxy-L-phenylalanine : oxygen oxido-reductase, EC1.14.18.1), a $Cu^{2+}$-containing enzyme, which catalyzes sequential oxidation of L-tyrosine to L-dihydroxyphenylalanine (DOPA), and DOPA to DOPA quinone. DOPA quinone subsequently autopolymerizes to generate melanin[2]. Intracellular and extracellular tyrosinase activities had been characterized in several *Streptomyces* species[3~5], and the study on the extracellular enzyme of *Streptomyces antibioticus* revealed *mel* operon consisting of two open reading frames (ORFs)[6]; the tyrosinase is encoded by the second ORF (*melC2*), while the first ORF (*melC1*) is assumed to encode a secretory protein involved in incorporation of $Cu^{2+}$ into the apoenzyme[7]. Introduction of *S. antibioticus mel* operon into *Streptomyces lividans*, a non-melanin-producing organism, on a high-copy-number plasmid conferred melanin production[6], suggesting that MelC2 tyrosinase activity is involved in the melanin synthesis. Accumulating data in the nucleotide sequence database have predicted the wide distribution of *mel* operon among *Streptomyces*.

We previously reported the marked stimulatory effect of $Cu^{2+}$ ion on morphological differentiation and antibiotic production in a bald mutant of *Streptomyces tanashiensis* as well as in the wild-type strains of a variety of *Streptomyces* species including *Streptomyces griseus*[8]. In many cases, the stimulatory effect of $Cu^{2+}$ on morphological and physiological differentiation was associated with enhanced production of melanin or melanin-like pigments, which led us to presume involvement of melanogenesis in the stimulation of differentiation. Here, to reveal the possible correlation between melanogenesis and differentiation processes, we isolated melanin negative mutants of *S. griseus* IFO13350, the model organism for the study of hormonal regulation by A-factor (2-isocapryloyl-3*R*-hydroxymethyl-$\gamma$-butyrolactone)[9]. We also conducted

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molecular cloning and mutational studies of a *mel* homologous gene of this organism to examine its role in differentiation.

## Materials and Methods

### Bacterial Strains, Plasmids and Media

The wild-type strain *S. griseus* IFO13350 and the A-factor-deficient mutant HH1 were described previously[9]. *Streptomyces lividans* TK21[10] was obtained from D. A. HOPWOOD. *Bacillus subtilis* ATCC6633 was used for the bioassay of streptomycin. *Escherichia coli* JM109 [*Δ(lac-pro) thi-1 endA1 gyrA96 hsdR17 relA1 recA1*/F′ *traD36 proAB lacI*$^q$ *lacZΔM15*] and pUC19[11] were used for general DNA manipulation. Media and growth conditions for *E. coli* were as described by MANIATIS *et al.*[12] Plasmid pIJ922 (carrying thiostrepton resistance) has a copy number of 1 to 2 per genome[10], and pIJ486 (carrying thiostrepton resistance[13]) and pIJ702 (carrying thiostrepton resistance and *melC1C2* of *S. antibioticus*[6]) have a copy number of 40~100. pKU206[14] was used for the disruption of *melC2* (see below). *S. griseus* strains were grown in Bennett's/glucose [containing (grams per liter): yeast extract (Difco Laboratories, Detroit, Mich.), 1; meat extract (Kyokuto, Tokyo, Japan), 1; *NZ* amine (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), 2; and glucose (Kokusan, Tokyo, Japan), 10, (pH 7.2)], YMP/glucose [containing (grams per liter): yeast extract (Difco), 2; meat extract (Kyokuto), 2; Bacto Peptone (Difco), 4; NaCl, 5; $MgSO_4/7H_2O$, 2; and glucose (Kokusan, Tokyo, Japan), 10, (pH 7.2)] and Nutrient Broth (NB; Difco). $CuSO_4$ (Kokusan) and *p*-methoxyphenol (Kokusan) were added at 10~700 $\mu$M and 0.6 mg/ml, respectively. Agar (Kokusan) was added at 1.5% for solid media. For the selection of transformants, ampicillin (Wako) at final concentration of 50 $\mu$g/ml was used for *E. coli*. For *S. griseus* transformants, thiostrepton (Sigma Chemical Company, St. Louis, Mo.) was added at final concentration of 20 $\mu$g/ml.

### Mutagenesis

To isolate melanin-negative mutants, *S. griseus* IFO13350 was mutagenized with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). Spores ($2.0\times10^9$) were incubated with 4 mg MNNG in 4 ml of 0.1 M Tris-maleate buffer (pH 9.0) for 90 minutes at 30°C with gentle shaking. Spores were then collected by centrifugation and washed 4 times with Tris-maleate buffer and plated onto YMP/glucose agar supplemented with 700 $\mu$M $CuSO_4$ after appropriate dilution. Plates were incubated at 30°C for 6 days and melanin-negative colonies were selected. To estimate streptomycin productivity of the mutants, NB soft agar containing *B. subtilis* spores was overlaid after 5 days growth, and incubated at 37°C overnight[9]. A-factor productivity was checked by the extracellular complementation of the streptomycin productivity in HH1 strain; each mutant colony examined was inoculated in close proximity to that of HH1 on YMP/glucose solid medium and incubated at 30°C. After 4 days growth, streptomycin production was detected by overlaying with *B. subtilis*. Strains that restored streptomycin production in HH1 were judged as A-factor positive[9].

### General Recombinant DNA Studies

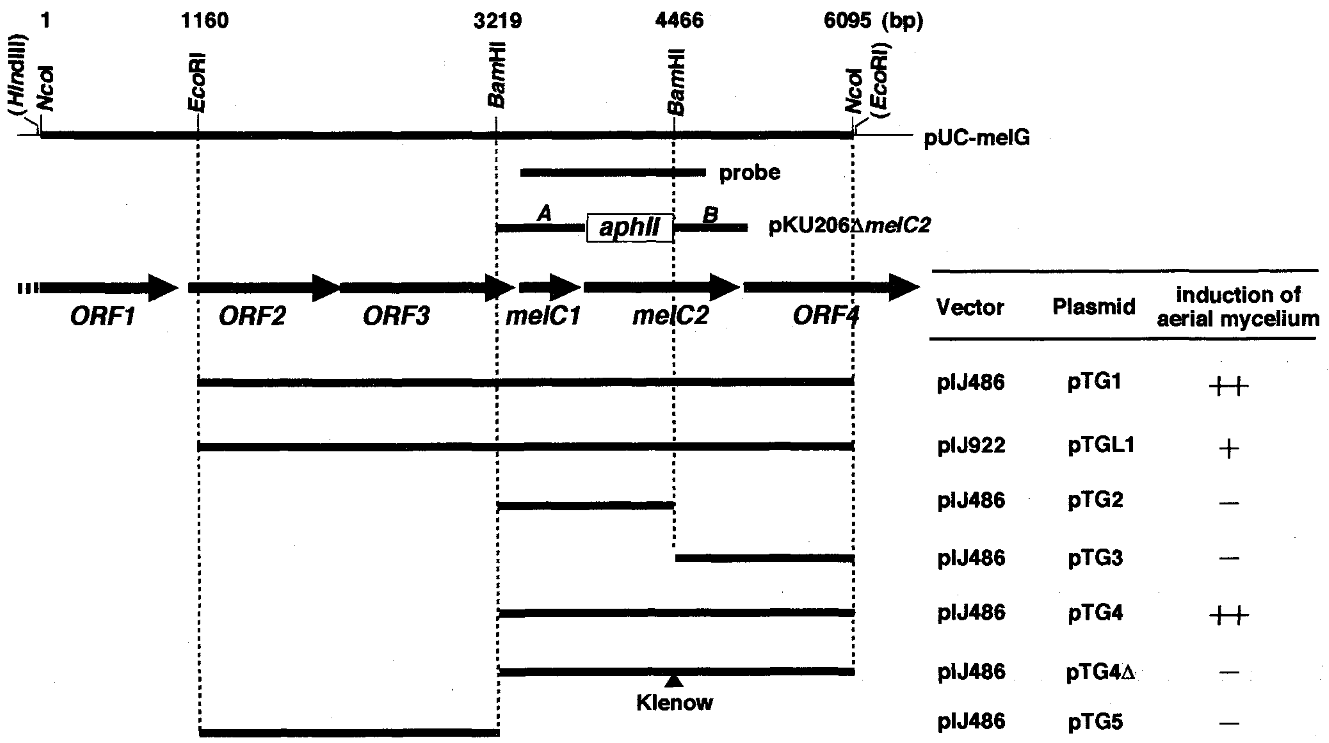
Restriction enzymes and other DNA-modifying enzymes were purchased from TaKaRa Shuzo, Kyoto, Japan. [$\alpha$-$^{32}$P]dCTP (110 TBq/mmol) for DNA labeling with a TaKaRa *Bca*Best DNA labeling system was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). DNA manipulation, Southern hybridization and colony hybridization were as described by MANIATIS *et al.*[12], and those in *Streptomyces* were as described by HOPWOOD *et al.*[10] Nucleotide sequence was determined by the dideoxy chain termination method with a Thermo Sequenase cycle sequencing kit (Amersham) on an automated DNA sequencer (LiCor model 4200, Lincoln, Nebr.).

### Cloning of *melC1C2* of *S. griseus*

To clone the *mel* homologue, the 5′ oligonucleotide 5′-GCCGGATCCCGCATGCC and the 3′ oligonucleotide 5′-GCCGGATCCAGAACACCGGGTCG (respectively corresponding to 264~274 and 1466~1483 nucleotides of *S. antibioticus melC1C2*[6]) were used in the standard PCR technique to amplify an internal segment of *melC1C2* of *S. griseus*. The resultant 1.2-kb amplicon was used as a probe in the following standard Southern hybridization and colony hybridization experiments to clone the 6.1-kb *Nco*I fragment containing *mel* and the flanking regions (Fig. 1). The *Nco*I fragment was cloned at the *Nco*I site of pUC-Nco to generate pUC-melG. pUC-Nco was created by attaching an 8-mer *Nco*I linker at the *Hinc*II site of pUC19.

The *Streptomyces* plasmids containing subcloned fragments were constructed as follows: to construct pTG1 and pTGL1, the 4.9-kb *Eco*RI-*Nco*I fragment was excised as an *Eco*RI fragment from pUC-melG and ligated at the *Eco*RI site of pIJ486 and pIJ922, respectively. To construct pTG2, the 1.2-kb *Bam*HI fragment from pUC-melG was inserted at the *Bam*HI site of pIJ486. To construct pTG3, the 1.6-kb *Bam*HI-*Nco*I fragment was excised as a *Bam*HI-*Eco*RI fragment from pUC-melG and inserted between the

Fig. 1. Restriction map of the 6.1-kb *Nco*I region containing *S. griseus melC1C2* and subcloned fragments.




| Vector | Plasmid | induction of aerial mycelium |
|---|---|---|
| pIJ486 | pTG1 | ++ |
| pIJ922 | pTGL1 | + |
| pIJ486 | pTG2 | − |
| pIJ486 | pTG3 | − |
| pIJ486 | pTG4 | ++ |
| pIJ486 | pTG4Δ | − |
| pIJ486 | pTG5 | − |

The fragments used for colony hybridization and gene disruption are shown together with the directions and extents of the ORFs deduced from the nucleotide sequence. The subcloned fragments on plasmids and their ability to induce aerial mycelium formation in *S. lividans* TK21 are also shown. The bracketed restriction sites are of the multicloning sites on pUC19.

*Bam*HI and *Eco*RI sites of pIJ486. pTG4 was constructed by inserting the 1.2-kb *Bam*HI fragment at the *Bam*HI site of pTG3. The correct orientation was confirmed by restriction mapping. To construct pTG4*Δ*, carrying the frame-shift mutation in *melC2*, pTG4 was partially digested with *Bam*HI followed by the blunt-end-formation with Klenow fragment and self-ligation. The correct construction was confirmed by restriction mapping. pTG5 was constructed by inserting the 2.1-kb *Eco*RI-*Bam*HI fragment from pUC-melG between the *Eco*RI-*Bam*HI sites of pIJ486.

## Gene Disruption

The chromosomal *melC2* gene was disrupted according to the procedure used for *amfC* disruption[15]. The disruption plasmid (pKU206*ΔmelC2*; Fig. 1) was constructed as follows: the two DNA fragments containing *melC2* flanking regions (fragment *A* and *B*) were amplified by the standard PCR using primers [5′-GCCGGATCCGGAGACCGTC] (corresponding to −821∼−805 when the adenine residue of the translation initiation codon of *melC2* was numbered as +1) and [5'-GGTAGATCTTGCGTACGTGGACCATCG] (−1∼+25) for fragment *A*, and primers [5′-CGA-AGATCTGGCCCTGAACCGGC] (+423∼+445) and [5′-GTCGGATCCGTCGAGAAGACGAGACC] (+1070∼+1095) for fragment *B*. The two amplicons were ligated after digesting with *Bgl*II, and the resultant ligated DNA was cleaved with *Bam*HI and inserted at the *Bam*HI site of pUC19. The plasmid was then digested with *Bgl*II and ligated to the 1.8-kb *Bam*HI fragment containing *aphII* gene[16]. Thus prepared 3.3-kb of mutated construct was excised as a *Bam*HI fragment and inserted at the *Bam*HI site of pKU206 to generate pKU206*ΔmelC2*. pKU206 is known to be unstable in *S. griseus* in the absence of thiostrepton used as a selection marker[15]. pKU206Δ*melC2* was introduced by transformation into *S. griseus* IFO13350, and a thiostrepton- and kanamycin-resistant transformant was then cultured at 30°C for 72 hours in YMP/glucose

liquid medium without thiostrepton and plated onto YMP/glucose agar plates containing 20 $\mu$g/ml kanamycin. Kanamycin-resistant colonies thus obtained were then checked for their sensitivity to thiostrepton and finally three colonies showing thiostrepton sensitivity and kanamycin resistance were obtained. All three colonies showed the same phenotype as described in the text. The correct recombination was confirmed by Southern hybridization using the 1.2-kb *Bam*HI fragment containing *melC1* and the 1.8-kb *Bam*HI fragment containing *aphII* as probes against the chromosomes digested with *Bam*HI, *Bam*HI plus *Hind*III, *Nco*I and *Fba*I. The hybridization profiles corresponding to each expected fragment length confirmed the true disruptant (Fig. 3).

Table 1. Phenotypes of melanin-negative mutants of *S. griseus*.

| Group | A-factor | Sm | aerial mycelium | number of isolates |
|---|---|---|---|---|
| I | – | – | – | 19 |
| II | + | + | + | 2 |
| III | + | + | – | 12 |
| IV | + | – | + | 4 |
| V | + | – | – | 1 |

## Results

### Isolation of Melanin-negative Mutants of *S. griseus*

To examine the possible correlation between melanogenesis and differentiation, MNNG-induced melanin-negative mutants of *S. griseus* were isolated. Mutants were screened on Bennett's/glucose solid medium supplemented with 700 $\mu$M of $CuSO_4$, since it remarkably enhanced melanin production in this organism. Among $6\times10^3$ mutagenized colonies, 38 mutants showing distinct and stable deficiency in melanin production were selected and classified into 5 groups as to the abilities to produce A-factor and streptomycin, and to form aerial mycelium (Table 1). A remarkable feature was that almost all the mutants (36/38) were deficient in aerial mycelium formation and/or streptomycin production. The most frequently isolated mutants classified as group I (19/38) was deficient both in streptomycin production and aerial mycelium formation with the loss of A-factor productivity. The mutants restored the parental phenotypes including melanogenesis by exogenous supply of synthetic A-factor. On the other hand, mutants belonging to groups III-V (17/38) showed deficiency in morphological differentiation and/or streptomycin production without lacking the A-factor-productivity.

### Cloning of *mel* and the Flanking Region of *S. griseus*

Above results prompted us to search and clone a homologue of *melC1C2*, the common *Streptomyces* tyrosinase gene, to examine its role in melanogenesis and differentiation of *S. griseus*. Primers hybridizing to the nucleotide sequences corresponding to the amino-terminus of MelC1 and the internal region of MelC2 of *S. antibioticus* were used in the standard PCR and the internal segment of the homologous gene was successfully amplified from *S. griseus* chromosomal DNA (see Materials and Methods). By using the amplicon as a probe, we then cloned the 6.1-kb *Nco*I fragment, which contained 4 complete (*ORF2*, *ORF3*, *melC1* and *melC2*) and 2 truncated ORFs (*ORF1* and *ORF4*; Fig. 1). MelC1 and MelC2 products of *S. griseus*, consisting of 127 and 306 amino acids, respectively, showed distinct homology to those identified in other *Streptomyces* species including *Streptomyces coelicolor* A3(2) (Fig. 2). MelC1 product possessed a potential signal peptide signature in the amino-terminal 25 amino acids. In MelC2, all the histidine residues for potential copper binding were conserved.

The amino-terminally truncated ORF1 product consisting of 319 amino acids showed distinct homology with tetracycline-transporting proteins. ORF2 (352 amino acids) and ORF3 (456 amino acids) products exhibited weak similarity to acid-coenzyme A ligases (EC6.2.1) and aldehyde dehydrogenases (EC1.2.1), respectively. The carboxy-terminally truncated ORF4 protein (291 amino acids) was homologous to a series of membrane transporter represented by BenK benzoate transporter of *Pseudomonas putida* (accession no. AF218267). Overall, the identified ORFs were likely to comprise a biosynthetic gene cluster. The gene organization of the *mel* flanking regions differed from those of other *Streptomyces*.

Fig. 2. Multiple alignment of MelC1 and C2 products.

**MelC1**

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GRI> MPMNRREMSCHHRGALAAAAAVPLLS-----GGEGEGAA----EAAAAPRS--------------------Q-RRGRSTPSAISAGPYG  59
ANT> MPELTRRRALG--AAAVVAAGVPLVA---LPAARADDR-----GHHT-PEVPGNPAASG------APAAFDEIYKGRRIQGRTVTDGGG  72
GLA> MPELSRRRALG--AAAALAAAAGTQA---VAAPAATAA-----GHHPGPSTAATGHHPG------TPASFDEVYKGRRIQGR--PAAGG  71
ALB> MPDITRRRAYT--TAAAVAATASAAAPTAAPAATAAAR-----HDHTAPDS------------------FDEVYKGRRIQGGP-ASGGG  63
LIN> MPRLTRRRALT--AAAALASGAGAGAGAQAAAAPGAAA-----HDHGSPDVPLPCSLLDLLPL--LPLLLDEVIKGRGIQGGP-HRGGG  79
TAN> MSSITRRRALG--VAAGAAGAAAGLA----LAGQAVAA-----PRAAAPAA--------------APASFDEVYQGRRIKGG--PSGGG  62
COE> PASGTRRQVMRGLFAPALAVGLAPLIAASRPSGAAEAATTGPLPAHPGPHAAESAESVEGAAEGVEGAAFDETYRGRRIRGIR-SAAGR 113
      . *.    *  *        . .        *               : :** .

GRI> SRS----------G-ERRRCVHRRPAAPHHEVRRRRLPQLHVPLRDGPQ-RALHAARRAVEEL-RGAALQPSTHGTHVTHL-------- 127
ANT> HHGGGHGGDGHGGGHHGGGYAVFVDGVELHVMRNADGSWISVVSHYEPVDTPRAAARAAVDEL-QGARLLPFPSN------------- 146
GLA> HH-----------QHHGGGYAVLIDGVELHVMQNADGSWISVVSHYDPVPTPRAAARAAVDEL-QGARLLPFPAN------------- 134
ALB> HHH-----------EHGGGYAVFVDGVQLHVMQNADGTWISVVSHYAPVATPRAAARAAVDEL-QGAPLLPFPTN------------- 126
LIN> HH-------------HGTGYAVFLDGVELHVMRNADGSWISVVSHYDPVPTPRAAARAAVDEL-QGAKLVPFPAN------------- 140
TAN> HHG----------GHHGGGYSVTIDGEELHVMQNADGTWISVINHYEPVATPKAVARAAVREL-QGAPLVPLTLA------------- 126
COE> AVG-------------AGTWQVTVDGRPLHLMRRADGSWLSMVDHYRSYPTPLAAARGAVDELGPGEHLRDTPSTDHGRHSGGRHGVHA 189
                     .  * ::.  . : :  :  .   .  .** ** **  *  *   .
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**MelC2**

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                                                  Copper A
GRI> MVHVRKNHLTMTAEEKRRFVHAVLEIKRRGIYDRFVKLHIQVNSTDYLDKESGKR--LGHVNPG-FLPWHRQYLLKFEQALQKVDPRV  85
ANT> -MTVRKNQASLTAEEKRRFVAALLELKRTGRYDAFVTTHNAFILG---DTDNGER--TGHRSPS-FLPWHRRFLLEFERALQSVDASV  81
GLA> -MTVRKNQATLTADEKRRFVAAVLELKRSGRYDEFVTTHNAFIIG---DTDAGER--TGHRSPS-FLPWHRRYLLEFERALQSVDASV  81
ALB> -MTVRKNQAALTADEKRRFVAAVLELKRNGRYDEFVRTHNEFIMS---DTRTGRRGGPGHRLPLPFLPWHRRFLLDFEQALQSVDSSV  84
LIN> -MTVRKNQATLTADEKRRFVTAVLSSS-AARYDTFVTTHNEFIVA---DTDNGER--TGHRSPS-FLPWHRRFLLEFERALQSVDASV  80
TAN> -MTVRKNQATLTADEKRRFVNALLELKRSGQYDTFVTTHNAFIMS---DTDNGDR--VGHRSPS-FLPWHRRFLIQFEQALQSVDATV  81
COE> MAYTRKDVSTLTRTEKRRFVNALLEIKRRGEYDEFVRTHIEYYVS---DGENGLR--TAHMAPS-FLPWHRRFLLDLEEALRRVDPSV  82
        .**:  ::*  ****** *:*. .  . ** ** *          *  * *   .*  *  *****::*:.:*.**: **. *

GRI> TLPYWDWTTDHGENSPLWSDTFMGGNGRPGDRRVMTGPFARRNG-WKLNISVIPEGPEDPALNRQLHPRRP--RLPRTGLRHAHPGPA 170
ANT> ALPYWDWSADRSTRSSLWAPDFLGGTGRSRDGQVMDGPFAASAGNWPINVRVD----GRTFLRRALGAGVS--ELPTR---------- 153
GLA> ALPYWDWSADRTARASLWAPDFLGGTGRSLDGRVMDGPFAASAGNWPINVRVD----GRAYLRRSLGTAVR--ELPTR---------- 153
ALB> ALPYWDWSTDRTVRASLWAPDFLGGTGRSSDGRVMDGPFAASTGNWPVNVRVD----GRTFLRRSLGTGVR--ELPTR---------- 156
LIN> ALPYWDWSTDRSARSSLWAPDFLGGTGRSRNGRVTDGPFRAATGVWPITVRLD----GRTYLRRALGGAGR--ELPTR---------- 152
TAN> TLPYWDWTADRTSRSSLWAPDFLGGTGRARDGQVTDGPFARTGNRWTINVRVD----GRDYLRRDLGAGGR--QLPTR---------- 153
COE> TVPYWDWTKDRSAKSAPWTADLLGGTGRRSDHRVTTGPFAHAGGNWTIKVNVT----DTEYLTRDLGRAADPLGLPTK---------- 156
     ::*****: *:  .:. *:  ::**.**  : :*  ***     . * :.: :           * * *        **
                                                                  Copper B
GRI> DPAELEQTLDLTVYDCPPWNHTSGGTPPYESFRNHLEGYTKFAWEPRLG----KLHGAAHVWTGGHMMYIGSPNDPVFFLNHCMIDRC 254
ANT> --AEVDSVLAMATYDMAPWNSGS------DGFRNHLEG-----W---RG---VNLHNRVHVWVGGQMATGVSPNDPVFWLHHAYIDKL 222
GLA> --AEVESVLGMATYDTAPWNSAS------DGFRNHLEG-----W---RG---VNLHNRVHVWVGGQMATGMSPNDPVFWLHHAYVDKL 222
ALB> --AEVDSVLSMATYDMAPYNSAS------DGFRNHLEG-----W---RG---VNLHNRVHVWVGGQMATGVSPNDPVFWLHHAYNRQL 225
LIN> --AEVDSVLSIPTYDMAPWNSAS------DGFRNHLEG-----W---RG---VNLHNRVHVWVGGQMATGVSPNDPVFWLHHAYIDKL 221
TAN> --AEVDSVLAMETYDMAPWNSSS------DGFRNHLEG-----W---RG---VNLHNRVHVWVGGQMATGVSPNDPVFWMHHAFVDKL 222
COE> --SDLEWALDDPKYDTSPYDSTVR-----KGFRNKLEG-----WGAGRGSVSWRNHNRVHRWVGGAMVGGASVNDPVFWLHHAFIDLQ 232
       :::: .*    ** .*::         ..***:***     *    *     . *. .* *.** *    * *****:::*.

GRI> WALWQARHPDVPHYLPTVP----TQD---VPDLNTPLGPWHTKTPADLLDHTRFYTYDQ-- 306
ANT> WAEWQRRHPSS-PYLPGG----GTPN---VVDLNETMKPWNDTTPAALLDHTRHYTFDV-- 273
GLA> WAEWQRRHPGS-GYLPAA----GTPD---VVDLNDRMKPWNDTSPADLLDHTAHYTFDTD- 274
ALB> WAEWQRRHPGA-GYVPTG----GTPD---VVDLNDTMKPWNDVRPADLLTHTAHYTFDV-- 276
LIN> WAQWQRRHRTP-AYVPAA----GTPD---VVDLDETMKPWHDSSPADLLDHTGHYTFDTD- 273
TAN> WADWQARHPRS-TYLPAA----GTAN---VVDLGDTMRPWNDVTPADMLDHTRHYTFDTAA 275
COE> WSRWQARHRGA-RYLPAEPPGRGSAQRGRIVARHEKLPPW-DVTPDELEDVGRIYRYA--- 288
     *: ** **     *:*      : :    :      : **    *  :      * :
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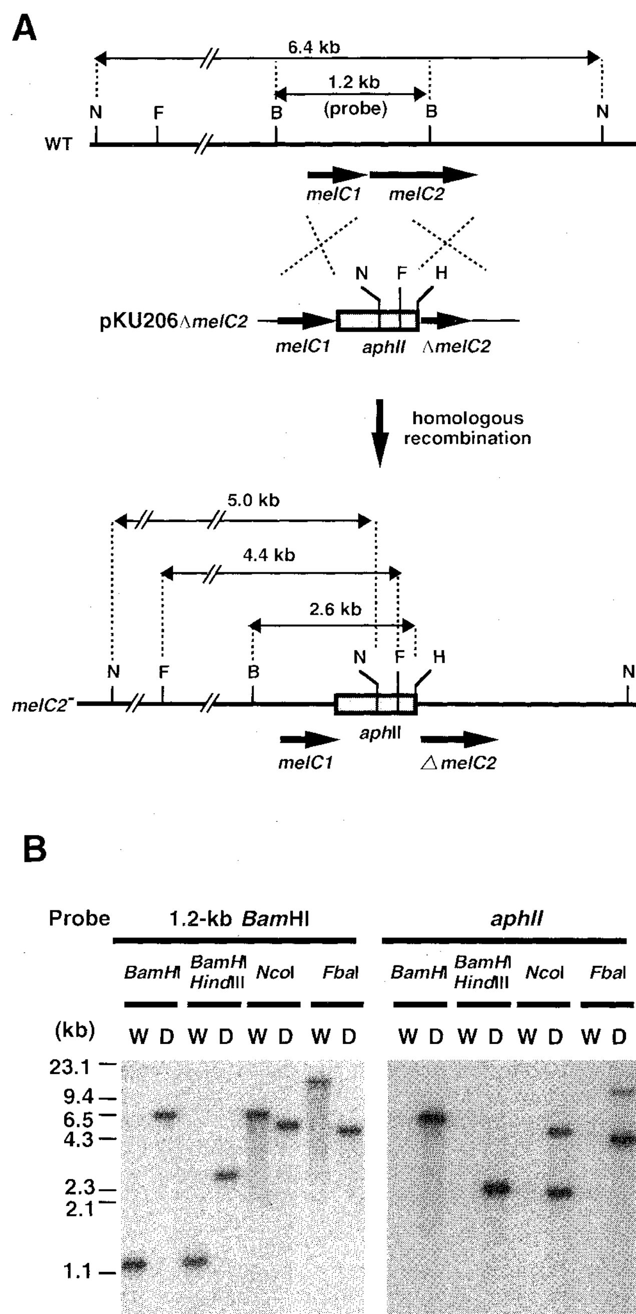
Sequences retrieved from the database were aligned by Clustal W program[19]. GRI, *S. griseus* (this study); ANT, *S. antibioticus* (accession no. M11582); GLA, *Streptomyces glaucescens* (Y00457); ALB, *Streptomyces albus* (X95705); LIN, *Streptomyces lincolnensis* (X95703); TAN, *S. tanashiensis* (AB052940; our unpublished work); COE, *S. coelicolor* A3(2) (AL356595). The histidine residues for potential copper binding are boxed.

### Effects of Gene Disruption and Introduction of *melC2*

To assess the involvement of the tyrosinase in melanin formation of *S. griseus*, the chromosomal *melC2* gene was disrupted. The wild-type *melC2* allele was replaced by the mutated construct with the insertion of kanamycin resistance gene by the standard homologous recombination technique (Fig. 3A), and the resultant kanamycin-resistant recombinant was checked for the true recombination by Southern hybridization analysis (Fig. 3B; see Materials and Methods). The resulted *melC2*-disruptant retained the parental level in melanin formation as well as in colony morphology and streptomycin production on YMP/glucose solid medium (Fig. 4A). However, the disruptant was significantly reduced in the production of a reddish pigment on the medium containing *p*-methoxyphenol, a putative analogue inhibitor for melanin biosynthesis in *Streptomyces*[17]. The pigment was probably a reaction product from the analogue by the activity of MelC2 tyrosinase that can replace melanin synthesis from tyrosine in *S. griseus*. This result indicated that MelC2 was expressed in an active form in the wild-type of *S. griseus*. The disruptant harboring pTGL1 (Fig. 1) showed the wild-type productivity of the pigment, which confirmed that the mutant phenotype was linked to the inactivation of *melC2*.

Further to examine the *mel*-related phenotypes, the high-copy-number plasmids carrying *mel* of *S. griseus* (pTG1; Fig. 1) and *S. antibioticus* (pIJ702[6]) were introduced into the representative melanin-negative mutants and wild-type of *S. griseus*. *S. lividans* TK21, an intrinsically non-melanin-producing organism, was also used as a host. While the introduction of pIJ702 conferred marked melanin formation, no plasmid carrying *S. griseus mel* caused apparent pigment production in any strains even in the presence of excessive $Cu^{2+}$ and tyrosine. Meanwhile, the introduction of pIJ702 and pTG1 caused precocious formation of aerial mycelium in *S. lividans* (Fig. 4B). The induction of aerial mycelium was more clearly observed when $Cu^{2+}$ was added to the media. The subcloning experiment with the high- and low-copy-number plasmids (summarized in Fig. 1) revealed that pTG4 but neither pTG4Δ nor pTGL1 retained the inducing activity, indicating that *melC2* on a high-copy-number plasmid was essential for the stimulation of aerial mycelium. A similar but ambiguous stimulation of aerial mycelium formation was observed with the wild-type and several group III mutants of *S. griseus* (not shown).

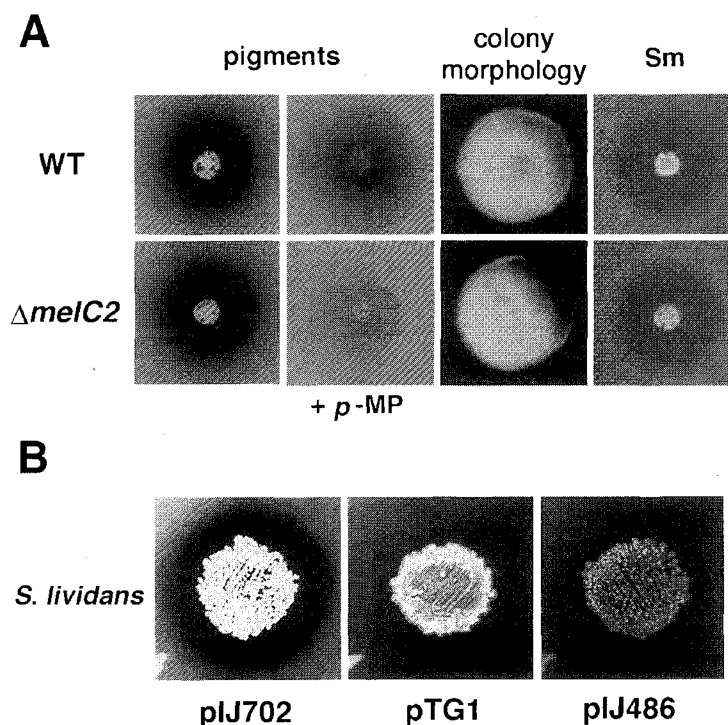
Fig. 3. Gene disruption of *melC2*.




(A) Schematic representation of the strategy used for disruption. pKU206Δ*melC2* carries the 3.3-kb *Bam*HI fragment containing the mutated *melC2* and the kanamycin resistance gene (*aphII*) on pKU206.

(B) Southern hybridization analysis against the chromosomal DNA from the wild-type and *melC2*-disruptant. The $^{32}P$-labelled 1.2-kb *Bam*HI fragment (containing *melC1* and the amino-terminal portion of *melC2*) and *aphII* gene cassette were used as probes.

Fig. 4. Phenotypes of *melC2*-disruptant and transformants harboring *mel* operon on plasmids.




(A) Colonies of the wild-type and *melC2*-disruptant of *S. griseus*. Each strain was inoculated on YMP/glucose agar supplemented with $Cu^{2+}$ (10 $\mu$M for sporulation and streptomycin production, and 700 $\mu$M for pigmentation) and cultured at 30°C. Patches were photographed after 4 days growth. Streptomycin production (Sm) was visualized by growth inhibition of *B. subtilis*. *p*-Methoxyphenol (*p*-MP) was added at 0.6 mg/ml.

(B) Colonies of *S. lividans* TK21 harboring pIJ702, pTG1 and pIJ486 (negative control). Strains were grown on YMP/glucose agar supplemented with 10 $\mu$M $Cu^{2+}$. Patches were photographed after 3 days growth.

## Discussion

The melanin-negative mutants of *S. griseus* isolated in this study mostly showed deficiency in the aerial mycelium formation and/or streptomycin production, which is consistent with the supposed correlation between melanogenesis and differentiation. About half of the mutants (group I) were deficient in the productivity of A-factor, which suggests that A-factor stimulates not only cellular differentiation and streptomycin production but also melanin formation in *S. griseus*. The mutants were probably generated by the loss of *afsA*[9], the gene involved in A-factor biosynthesis. This gene is frequently lost along with the deletion at the right terminal region of the linear chromosome[18]. On the other hand, the mutants of groups III~V were deficient in aerial mycelium formation and/or streptomycin production without the loss of A-factor-productivity. Although we need to localize the mutational points, the phenotypes of the mutants might reflect a role of melanogenesis in morphological and physiological differentiation in *S. griseus*.

This study also characterized the MelC1C2 homologue in *S. griseus*. Although the sequence homology was evident, the similarity ratio to the other *Streptomyces* counterparts was relatively low; *S. griseus* MelC1 carried the region with weak similarity in the carboxy-terminal half (21% in 65 amino acids, in comparison with *S. antibioticus* sequence). MelC2 was relatively larger than the other homologues due to the additional amino acid residues at the middle of the molecule. Those structural differences may cause altered activity or substrate specificity resulting in the apparent inability to confer melanin production, whereas the enzyme probably possesses the activity to oxidize *p*-methoxyphenol to produce the reddish pigment. MelC2

tyrosinase may have a higher affinity for *p*-methoxyphenol than for L-tyrosine, and this could be the reason for the effective inhibitory effect of the substance against melanogenesis in *Streptomyces*[17].

The involvement of phenol oxidases including tyrosinase in morphological differentiation has been suggested in fungi; several mutants defective in phenol oxidase activities are known to simultaneously lack the ability to perform cellular development (reviewed by BELL & WHEELER[2]). This study suggests that tyrosinase has a stimulatory activity on morphogenesis in *Streptomyces*. The introduction of *mel* gene on a high-copy-number plasmid probably caused overexpression of MelC2 tyrosinase that led to the acceleration of aerial mycelium formation. The stimulation was evident in *S. lividans*, possibly because the organism is intrinsically defective or reduced in the phenol-oxidizing enzyme activity. In *S. griseus*, neither the introduction of *mel* on plasmids nor the inactivation of *mel* resulted in clear phenotypic alterations. It does not necessarily exclude the involvement of MelC2 tyrosinase but strongly suggests another phenol oxidizing enzyme that plays a significant role in melanin formation and possibly in differentiation of this organism. We will report elsewhere identification of a novel phenol oxidase in *S. griseus*.

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