

GriC and GriD Constitute a Carboxylic Acid Reductase Involved in Grixazone Biosynthesis in *Streptomyces griseus*

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Abstract In grixazone biosynthesis by *Streptomyces griseus*, a key intermediate 3-amino-4-hydroxybenzoic acid (3,4-AHBA) is converted to another key intermediate 3-amino-4-hydroxybenzaldehyde (3,4-AHBAL). Two genes *griC* and *griD* in the grixazone biosynthesis gene cluster were found to be responsible for this conversion, because disruption of each gene resulted in the extracellular accumulation of 3-acetyl-amino-4-hydroxybenzoic acid, a shunt product from 3,4-AHBA. Significant sequence similarity of GriC to AMP-binding proteins and of GriD to NAD(P)-dependent aldehyde dehydrogenases suggested that GriC and GriD constituted an ATP- and NAD(P)-dependent carboxylic acid reductase (CAR) catalyzing reduction of 3,4-AHBA to produce 3,4-AHBAL through acyl-AMP formation, as is found for the reactions catalyzed by some CARs. *griG* encoding a benzoate transporter homologue in the grixazone biosynthesis gene cluster was nonessential for grixazone biosynthesis but probably enhanced the membrane permeability for 3,4-AHBA. Simultaneous overexpression of *griC*, *griD*, and *griG* in *S. griseus* mutant cells deficient in an acetyltransferase responsible for *N*-acetylation of 3,4-AHBA led to efficient bioconversion of exogenously added 3,4-AHBA to 3,4-AHBAL. This system also turned out to be useful for reduction of some aryl carboxylates to the corresponding aryl aldehydes.

Keywords *Streptomyces griseus*, grixazone, biosynthesis, carboxylic acid reductase, bioconversion, 3-amino-4-hydroxybenzoic acid, benzoate transporter

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Introduction

The filamentous, soil-inhabiting, Gram-positive bacterial genus *Streptomyces* is characterized by its ability to produce a wide variety of secondary metabolites including antibiotics and biologically active substances. We have studied the A-factor regulatory cascade that leads to secondary metabolite formation and morphological differentiation in *Streptomyces griseus* [1]. Grixazone, consisting of grixazone A (see Fig. 2 for its structure) and grixazone B (in which the aldehyde group of grixazone A is replaced by a carboxyl group) showing a parasiticide activity [2], is one of the secondary metabolites under the control of A-factor [3]. The grixazone biosynthesis gene cluster contains 13 genes [4], including *griI-griH* and *griE-griF* (Fig. 1A). *griI* and *griH* encode an aldolase and 3-dehydroquinase synthase homologues, respectively. *griE-griF* are homologous to *melC1-melC2* of streptomycetes, in which *melC1* and *melC2* encode a tyrosinase cofactor and a tyrosinase, respectively. We previously characterized these four genes and revealed two enzymatic reactions involved in grixazone biosynthesis (see Fig. 2), both of which had been unpredictable only on the basis of protein homology. One is the GriI-GriH reaction responsible for the formation of 3-amino-4-hydroxybenzoic acid (3,4-AHBA) from two primary metabolites, L-aspartate-4-semialdehyde and dihydroxyacetone phosphate [5]. The other is the GriE-GriF reaction responsible for the formation of the phenoxazinone chromophore of grixazones from two molecules of 3-amino-4-hydroxybenzaldehyde (3,4-AHBAL) [6]. These findings predicted that an enzyme(s) catalyzing the reduction of 3,4-AHBA to produce 3,4-AHBAL might be involved in grixazone biosynthesis and the enzyme gene(s) would be encoded in the gene cluster.

In this study, we identified *griC* and *griD* responsible for reduction of 3,4-AHBA to produce 3,4-AHBAL. *griC* encodes an AMP-binding protein, and *griD* encodes a protein exhibiting sequence similarity to some aldehyde dehydrogenases. Although *griC-griD* homologues have been found in other gene clusters, including those for biosynthesis of phenazine antibiotic D-alanylgriseoliteic acid in *Erwinia herbicola* [7] and a β -lactam antibiotic carbapenem, thienamycin, in *Streptomyces cattleya* [8], their functions have not been elucidated. Here we report that GriC-GriD are essential for grixazone biosynthesis and constitute a carboxylic acid reductase that reduces 3,4-AHBA to 3,4-AHBAL. In addition to the GriC-GriD function, we found that *griG* encoding a benzoate transporter homologue in the grixazone biosynthesis gene cluster had an apparently important role for efficient import of exogenously added 3,4-AHBA, although it was nonessential for grixazone biosynthesis.

Materials and Methods

Strains, Plasmids, Media, and Chemicals

S. griseus IFO13350 was obtained from the Institute of Fermentation, Osaka, Japan. A *nataA* null mutant ($\Delta nataA$) of *S. griseus* was constructed previously [9]. *S. griseus* strains were grown at 30°C in YPD medium (0.2% yeast extract, 0.4% Bacto peptone, 0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 1.0% glucose, and 0.5% glycine, pH 7.2) or at 26.5°C in standard minimal medium [SMM; 0.9% glucose, 0.9% asparagine, 0.2% $(NH_4)_2SO_4$, 0.24% Trizma base, 0.1% NaCl, 0.05% K_2SO_4 , 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2$, 2.5 mM KH_2PO_4 , and 1.0% trace element solution [10], pH 7.2]. For grixazone production, *S. griseus* cells were cultured for 5 days in SMM, the KH_2PO_4 concentration of which was 0.25 mM to allow expression of the grixazone biosynthesis genes [4]. We referred to the standard (2.5 mM KH_2PO_4) and low-phosphate (0.25 mM KH_2PO_4) SMM media as SMM-2.5 and SMM-0.25, respectively. SMM-2.5 was used for bioconversion experiments. A thiostrepton resistance plasmid pIJ702 containing the *melC* promoter [11], with its copy number of 40 to 300 [10], was used for DNA manipulation and gene expression in *S. griseus*. The *melC* promoter is active in the late exponential and stationary growth phases. *Escherichia coli* strains JM109, JM110, TOP10 (Invitrogen), and BL21 [pGro7] (Takara Biochemicals) and plasmids pUC19, pCR4Blunt-TOPO (Invitrogen), and pCold III (Takara Biochemicals) were used for DNA manipulation in *E. coli*. Restriction enzymes, T4 DNA ligase, *Pyrobrest* DNA polymerase, and other DNA-modifying enzymes were purchased from

Takara Biochemicals. 3,4-AHBAL, 3-acetylamino-4-hydroxybenzoic acid (3,4-AcAHBA), and 3-acetylamino-4-hydroxybenzaldehyde (3,4-AcAHBAL) were prepared as described previously [5, 6]. Other chemicals were purchased from Wako Chemicals. The absence of undesired alterations during PCR was checked by nucleotide sequencing.

Gene Disruption

The chromosomal *griC*, *griD*, and *griG* genes of *S. griseus* IFO13350 were disrupted as follows (Fig. 1A). Correct disruption was checked by Southern hybridization (data not shown). A 1.1-kb fragment containing a 3' portion of *griC* and its downstream region was amplified by PCR with primers, 5'-TGGGAATTCGTCTACTGATGAGCACCGT-CGCAC-3' (with an *EcoRI* site shown by underlining and the stop codon of *griC* shown in italics) and 5'-CAC-GACGGTCTCCGGATCCCC-3' (with an inherent *BamHI* site shown by underlining). The amplified fragment was digested with *EcoRI* and *BamHI*, ligated with a 1.3-kb *BamHI* fragment containing a further downstream region from *griC*, and placed between the *EcoRI* and *BamHI* sites of pUC19. From the resulting plasmid, the 2.4-kb *EcoRI-HindIII* fragment was excised and connected with a 3.4-kb *BglIII-EcoRI* fragment containing a 5' portion of *griC* and

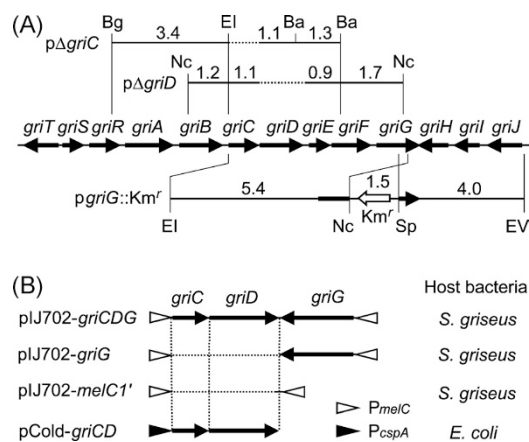


Fig. 1 Schematic representation of the DNA fragments on plasmids used in this study.

(A) Gene organization of the grixazone biosynthesis gene cluster [4] and DNA fragments on plasmids used for gene disruption. The restriction enzyme abbreviations: Ba, *BamHI*; Bg, *BglIII*; El, *EcoRI*; EV, *EcoRV*; Nc, *NcoI*; and Sp, *SphI*. Km^r represents the kanamycin/neomycin resistance gene from Tn5. Numerals indicate the length of DNA fragments in kb. The Km^r cassette in $p\Delta griC$ and $p\Delta griD$ is not shown. (B) DNA fragments on plasmids used for gene expression in *S. griseus* [$pIJ702-griCDG$, $pIJ702-griG$, and $pIJ702-melC1'$] and in *E. coli* [$pCold-griCD$]. P_{melC} , the *melC* promoter; P_{cpsA} , the *cspA* promoter.

its upstream region. This 5.8-kb fragment, which had a 1.0-kb in-frame deletion in *griC* (from Asp-10 to Gly-350 of GriC), was placed between the *Bam*HI and *Hind*III sites of pUC19, together with a 1.1-kb *Hind*III fragment carrying the kanamycin/neomycin resistance gene from Tn5, resulting in p Δ *griC*. The plasmid was alkali-denatured and introduced by protoplast transformation into *S. griseus* IFO13350. Neomycin (5 μ g/ml)-resistant transformants containing p Δ *griC* in the chromosome, as a result of a single crossover, were first isolated, and one of the transformants was cultured several times on YPD medium without neomycin. A neomycin sensitive colony was isolated as the true *griC*-disruptant (mutant Δ *griC*).

A 1.1-kb fragment containing a 5' portion of *griD* and its upstream region was amplified by PCR with primers, 5'-CGATGATCCCGAATTCGACAGTC-3' (with an inherent *Eco*RI site shown by underlining) and 5'-TGGTCTAGAGTGCGACGGTGCTCATCAGTAGAC-3' (with an *Xba*I site shown by underlining and the start codon of *griD* shown in italics). The amplified fragment was digested with *Eco*RI and *Xba*I, and ligated with a 1.2-kb *Nco*I-*Eco*RI fragment containing a further upstream region from *griD*. This 2.3-kb *Nco*I-*Xba*I fragment was placed between the *Hind*III and *Xba*I sites of pUC19 using a short linker composed of *Hind*III-*Sph*I-*Pst*I-*Sal*I-*Xba*I-*Bam*HI-*Sma*I-*Nco*I sites. From the resulting plasmid, a 2.3-kb *Xba*I fragment was excised and used for further plasmid construction. On the other hand, a 0.9-kb fragment containing a 3' portion of *griD* and its downstream region was amplified by PCR with primers, 5'-TGGTCTAGACCCGGCGCTGACCCCTCCCG-3' (with an *Xba*I site shown by underlining and the stop codon of *griD* shown in italics) and 5'-TCAGGGCCGGATCCTCGGGG-3' (with an inherent *Bam*HI site shown by underlining). The amplified fragment was digested with *Xba*I and *Bam*HI, and ligated with a 1.7-kb *Bam*HI-*Nco*I fragment containing a further downstream region from *griD*. This 2.6-kb *Xba*I-*Nco*I fragment was placed between the *Xba*I and *Eco*RI sites of pUC19 using a short linker composed of *Nco*I-*Sma*I-*Kpn*I-*Sac*I-*Eco*RI sites. At the *Xba*I site of the resulting plasmid, the 2.3-kb *Xba*I fragment described above was placed in the correct orientation. This plasmid contained a deleted *griD* sequence, encoding a hexapeptide of the first 5 amino acids of GriD (Met-Ser-Thr-Val-Ala) followed by Leu, with the 2.3-kb upstream and 2.6-kb downstream regions from *griD*. Finally, a 1.1-kb *Hind*III fragment carrying the kanamycin/neomycin resistance gene from Tn5 was placed at the *Hind*III site of the plasmid, resulting in p Δ *griD*. By use of this plasmid, a *griD*-disruptant (mutant Δ *griD*) was similarly isolated by the method used for obtaining the *griC*-disruptant.

A 5.4-kb *Eco*RI-*Nco*I fragment containing a 5' portion

of *griG* and its upstream region was connected with a 1.5-kb *Sal*I-*Hind*III fragment carrying the kanamycin/neomycin resistance gene from Tn5, mediated by a short linker composed of *Nco*I-*Sma*I-*Kpn*I-*Sma*I-*Bam*HI-*Xba*I-*Sal*I sites, to give a 6.9-kb *Eco*RI-*Hind*III fragment. A 4.0-kb *Sph*I-*Eco*RV fragment containing a 3' portion of *griG* and its downstream region was connected with an *Sph*I linker and placed in the *Sph*I site of pUC19. From the resulting plasmid, a 4.0-kb *Hind*III-*Pst*I fragment was excised and placed between the *Eco*RI-*Pst*I sites of pUC19, together with the 6.9-kb *Eco*RI-*Hind*III fragment described above, resulting in p*griG*::Km^r. In this construct, *griG* was separated to two fragments that overlapped partially, from the 5'-terminus to position +1030 (corresponding to the *Nco*I site) and from position +857 (corresponding to the *Sph*I site) to the 3'-terminus, by the kanamycin/neomycin resistance gene inserted. The plasmid, p*griG*::Km^r, was digested with *Dra*I, alkali-denatured, and introduced by protoplast transformation into *S. griseus*. A neomycin-resistant transformant, as a result of double crossover between the disrupted *griG* sequence and the intact chromosomal *griG* sequence, was isolated as the true *griG*-disruptant (mutant *griG*::Km^r).

Construction of Plasmids

A 0.5-kb fragment containing the *melC* promoter and the 5'-portion (173 bp) of *melC1* was amplified by PCR using pIJ702 as a template and primers, 5'-CTGCAGAAGCTTGAATTCTGATCACGTCAGTTTTTC-3' (with a *Hind*III site and an *Eco*RI site shown by underlining) and 5'-AAGCTTCTGCAGTTGTAGATCTCGTCGAAG-3' (with a *Pst*I site and an inherent *Bg*III site shown by underlining). The amplified fragment, which contained an *Sph*I site (GCATGC) overlapping with the start codon of *melC1* shown by underlining, was digested with *Hind*III plus *Pst*I and cloned into pUC19, resulting in pUC702. This plasmid was used for the construction of expression plasmids in *S. griseus*, pIJ702-*griG* and pIJ702-*griCDG*.

The 0.5-kb *Eco*RI-*Bg*III fragment excised from pUC702 was placed between the *Bg*III and *Eco*RI sites of a pIJ702-derived plasmid, in which the *melC1*-*melC2* sequence under the *melC* promoter was replaced by a short linker containing *Sph*I, *Bg*III, and *Eco*RI sites, resulting in pIJ702-*melC1'* (Fig. 1B). This plasmid was used as a negative control plasmid. The *griG* sequence was amplified by PCR using primers, 5'-CATGTACGCAGTACTCGCC-3' (with the start codon of *griG* shown in italic) and 5'-CTCGAGAGATCTTCAACGAGTTCTGCCTG-3' (with the stop codon of *griG* shown in italic and a *Bg*III site shown by underlining). The amplified fragment was digested with *Bg*III and placed in pUC702, which had been digested with

SphI, blunted with T4 DNA polymerase, and digested with *BglII*. From the resulting plasmid, a 1.7-kb *EcoRI*-*BglII* fragment containing the *griG* sequence under the *melC* promoter was excised and placed between the *EcoRI* and *BglII* sites of the pIJ702-derived plasmid, resulting in pIJ702-*griG* (Fig. 1B).

The inherent co-translational *griC*-*griD* sequence was amplified by PCR using primers, 5'-GCATGCTCCTC-ATCGATGATC-3' (with the start codon of *griC* shown in italic and an *SphI* site shown by underlining) and 5'-AGATCTTCAGCGCCGGCCACGAC-3' (with the stop codon of *griD* shown in italic and a *BglII* site shown by underlining). After the amplified fragment had been cloned into pCR4Blunt-TOPO, the *griC*-*griD* sequence was excised with *SphI* plus *BglII* and cloned into the pIJ702-derived plasmid. Between the *BglII* and *NdeI* sites of the resulting plasmid, a 2.7-kb *BglII*-*NdeI* fragment containing the *griG* sequence under the *melC* promoter from pIJ702-*griG* were placed, resulting in pIJ702-*griCDG* (see Fig. 1B).

The *griC*-*griD* sequence was amplified by PCR using primers, 5'-GGAATTCCATATGAGCCTCATCGATG-3' (with the start codon of *griC* shown in italic and an *NdeI* site shown by underlining) and 5'-GCTCGAGTTAGC-GCCGGCCACGAC-3' (with the stop codon of *griD* shown in italic and an *XhoI* site shown by underlining). After the amplified fragment had been cloned into pCR4Blunt-TOPO, the *griC*-*griD* sequence was excised with *NdeI* plus *XhoI* and cloned into pCold III for expression in *E. coli*, resulting in pCold-*griCD*. On this plasmid, *griC* and *griD* were under the control of the *cspA* promoter that is inducible by cold-shock.

Bioconversion Conditions

S. griseus cells were precultured at 30°C for 2 days in 100 ml of YPD liquid medium supplemented with 10 µg/ml thiostrepton. The mycelium (about 4 g wet weight) was harvested by centrifugation, washed twice, resuspended in 10 ml of SMM-2.5, and homogenized. A portion (100 µl) of the homogenized solution was inoculated to 100 ml of fresh SMM-2.5 and cultured at 26.5°C for 2 days (up to the late exponential phase), and then an aryl carboxylic acid was added at a final concentration of 10 mM. After the cells were further incubated for 1~3 days, compounds in the culture broth were analyzed by reversed-phase high-performance liquid chromatography (HPLC), as described previously [6]. The aryl aldehydes produced were determined by liquid chromatography-electrospray ionization mass spectrometry, as described previously [5], in reference to the authentic compounds available commercially. The amounts of the aryl aldehydes produced were determined

by HPLC in reference to the authentic compounds.

The conditions for the conversion of 3,4-AHBA to 3,4-AHBAL in *E. coli* were as follows. The cells of *E. coli* BL21 [pCold-*griCD* and pGro7] ([] denotes plasmid-carrier state) were incubated at 37°C to an optical density 0.6 at 600 nm in LB medium supplemented with 50 µg/ml ampicillin and 20 µg/ml chloramphenicol, and then placed in a water bath at 15°C for 30 minutes. Isopropyl β-D-thiogalactopyranoside and L-arabinose were then added at final concentrations of 0.1 mM and 0.5 mg/ml, respectively, to allow the expression of *griC*-*griD* and the chaperon gene on pGro7. At the same time, 3,4-AHBA was also added at a final concentration 1 mM and the culture was further continued at 15°C for 24 hours.

Results and Discussion

GriC and GriD Are Both Required for Reduction of 3,4-AHBA to 3,4-AHBAL

On the basis of our findings on grixazone biosynthesis [5, 6], we assumed that a reductase catalyzing the reduction of 3,4-AHBA to 3,4-AHBAL should be encoded in the grixazone biosynthesis gene cluster. *griD* was the most probable candidate for the reductase gene, because GriD showed amino acid sequence similarity (about 30% identity) to some NAD(P)-dependent aldehyde dehydrogenases. The start codon of *griD* overlapped with the stop codon of *griC*, suggesting that these two genes were co-transcribed and co-translated. GriC showed sequence similarity to AMP-binding proteins, including the N-terminal AMP-binding domain of a CAR of *Nocardia* sp. [12, 13]. The *Nocardia* CAR has a reductase domain in the C-terminal portion and catalyzes the reduction of aryl carboxylic acids to the corresponding aldehydes through acyl-AMP formation, followed by NADPH-dependent reduction of the intermediate [12, 13]. This suggests that the *Nocardia* CAR is like a GriC-GriD fusion protein. The computer search predicted that GriC and GriD were functionally related, since GriC and GriD homologues are encoded adjacently on the chromosomes of various bacteria, such as *SCO7115-SCO7114* in *Streptomyces coelicolor* A3(2) [14], *thnN-thnO* in *S. cattleya* [8], *MAP3493-MAP3494* in *Mycobacterium avium* [15], *STH579-STH578* in *Symbiobacterium thermophilum* [16], *ehpF-ehpG* in *E. herbicola* [7], and *Plu3307-Plu3306* in *Photobacterium luminescens* [17] (the anterior genes encode GriC homologues and the posterior genes encode GriD homologues). We therefore expected that GriC and GriD would constitute a carboxylic acid reductase (Fig. 2).

To reveal the functions of GriC and GriD in the

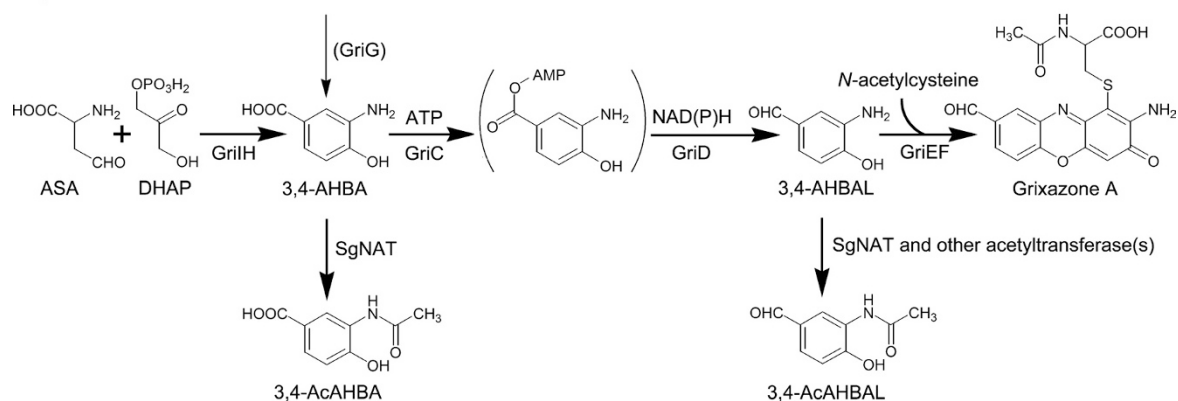


Fig. 2 The proposed biosynthetic pathway leading to grixazone A and shunt products, 3,4-AcAHBA and 3,4-AcAHBAL.

Grixazone A is synthesized through the following three steps: (i) 3,4-AHBA production from two primary metabolites by GriI and GriH [5], (ii) the reduction of 3,4-AHBA to 3,4-AHBAL by GriC-GriD (this study), and (iii) the formation of grixazone A in the presence of *N*-acetylcysteine by GriE and GriF [6]. A hypothetical pathway for reduction by GriC and GriD, on the basis of the sequence homology of GriC/GriD with the CAR of *Nocardia* [12], is also shown. GriG is nonessential for grixazone biosynthesis but apparently contributes the uptake of 3,4-AHBA excreted and/or leaked from the cells (this study). 3,4-AcAHBA is produced from 3,4-AHBA by a cytosolic enzyme, SgNAT, encoded by *nata* in *S. griseus* [9]. SgNAT is also responsible for *N*-acetylation of 3,4-AHBAL to produce 3,4-AcAHBAL, but other acetyltransferase(s) must be involved in the *N*-acetylation (this study). ASA, L-aspartate 4-semialdehyde; and DHAP, dihydroxyacetone phosphate.

grixazone biosynthesis, we constructed *griC*- and *griD*-disrupted strains. Mutant Δ *griC* has an in-frame deletion of the region corresponding to Asp-10 to Gly-350 of GriC. In mutant Δ *griD*, the chromosomal *griD* sequence corresponding to Pro-6 to Ala-454 was replaced by a short linker, resulting in production of only the first five amino acids of GriD (Met-Ser-Thr-Val-Ala), followed by a Leu. As we expected, Δ *griC* and Δ *griD* mutants produced no grixazone and accumulated detectable amounts of 3-acetylamino-4-hydroxybenzoic acid (3,4-AcAHBA) in the culture broth, when grown in SMM-0.25 at 26.5°C for 5 days (Fig. 3A). SMM-0.25 contained a low concentration (0.25 mM) of KH_2PO_4 to allow expression of the grixazone biosynthesis genes, because the *gri* genes are expressed under phosphate-depleted conditions [4]. Because 3,4-AcAHBA is a shunt product derived from 3,4-AHBA by an arylamine *N*-acetyltransferase (SgNAT) encoded by *nata* in *S. griseus* [9], these observations implied that both mutants accumulated 3,4-AHBA. Note that both 3,4-AHBA and 3,4-AcAHBA were never detected in the culture broth of the wild-type *S. griseus* cells under grixazone-producing conditions [6]. The defect of grixazone production in mutants Δ *griC* and Δ *griD* was complemented by introduction of a low-copy number plasmid containing the intact *griC-griD* sequence with the *griC* promoter (data not shown).

We next examined bioconversion of 3,4-AHBA to 3,4-AHBAL by *E. coli* BL21 [pGro7] harboring pCold-*griCD* that contained a co-translational *griC-griD* sequence under

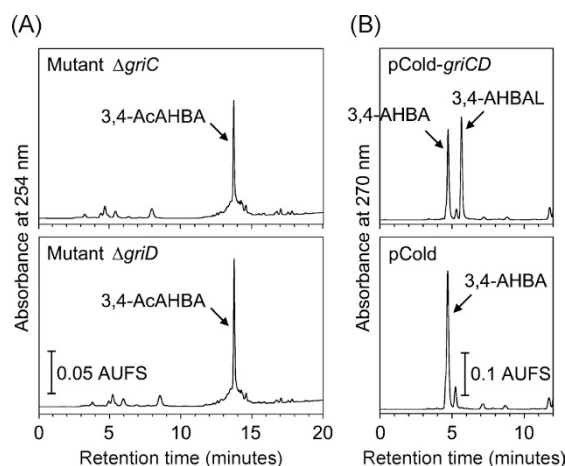


Fig. 3 HPLC analysis of culture broths of *S. griseus* mutants Δ *griC* and Δ *griD* (A) and *E. coli* BL21 [pCold-*griCD* and pGro7] grown in the presence of 1.0 mM 3,4-AHBA (B).

Authentic samples were used to identify the compounds. (A) Mutants Δ *griC* and Δ *griD* were cultured at 26.5°C for 5 days in SMM-0.25 and 10 μ l of the culture broths were analyzed by HPLC. (B) *E. coli* cells were grown at 15°C for 24 hours in LB medium supplemented with 1 mM 3,4-AHBA and 10 μ l of the culture broth was analyzed by HPLC. *E. coli* BL21 [pCold III and pGro7] was used as a negative control. AUFS in (A) and (B), absorbance units full scale.

the control of the cold-shock inducible *cspA* promoter on pCold III. When the production of GriC and GriD was initiated, 3,4-AHBA (final concentration, 1 mM) was added

to the culture. For 24 hours incubation at 15°C, 3,4-AHBA added was converted to 3,4-AHBAL with an efficiency of 46% (Fig. 3B, upper panel). This conversion was not observed in the control strain harboring the empty vector (Fig. 3B, lower panel). These observations suggested that GriC and GriD cooperatively reduced the carboxyl group of 3,4-AHBA to produce 3,4-AHBAL. This enzymatic reaction filled a gap between 3,4-AHBA and 3,4-AHBAL in the grixazone biosynthesis pathway (Fig. 2). The GriC-GriD function as a carboxylic acid reductase implies that GriC-GriD homologues, including EhpF-EhpG encoded in the phenazine biosynthesis gene cluster of *E. herbicola* [7] and ThnN-ThnO encoded in the thienamycin biosynthesis gene cluster of *S. cattleya* [8], are responsible for the reduction of a carboxylic acid to the corresponding aldehyde in the respective biosynthesis, as a novel reductase family.

A Proposed Mechanism of Catalysis by GriC and GriD

GriC shows similarity in amino acid sequence to AMP-binding proteins. GriD shows similarity to NAD(P)-dependent aldehyde dehydrogenases. The combination of GriC and GriD appears to constitute a reductase similar to the *Nocardia* CAR having the *N*-terminal AMP-binding domain and the *C*-terminal reductase domain [12, 13]. The ATP- and NADPH-dependent reduction of a carboxylic acid to the corresponding aldehyde *via* an acyl-AMP intermediate has also been found for a long-chain fatty acid reductase complex of luminescence bacteria [18]. On the basis of the knowledge for these enzymes [12, 13, 18], GriC and GriD presumably form a reductase complex and catalyze the reduction as follows: (i) GriC converts 3,4-AHBA to an acyl-AMP intermediate, and (ii) GriD reduces the intermediate to 3,4-AHBAL using NAD(P)H (Fig. 2). To clarify the reaction pathway, it is necessary to establish the reductive reaction from 3,4-AHBA to 3,4-AHBAL *in vitro* by using GriC and GriD proteins. Our repeated attempts to show the reaction *in vitro* have so far been failed, even by using cell-free extracts of the *E. coli* BL21 [pCold-*griCD* and pGro7] and *S. griseus* Δ *griG* [pIJ702-*griCDG*] (see below). This may be ascribed to low stability of GriC and/or GriD.

Possible Roles of *griG* in Grixazone Biosynthesis

GriG shows sequence similarity (about 30% identity) to the aromatic acid/H⁺ symporter family members [19]. To determine the role of GriG in grixazone biosynthesis, *griG* on the chromosome of *S. griseus* was disrupted by inserting the kanamycin/neomycin resistance gene within its coding region. The cells were incubated in SMM-0.25 for 5 days. In contrast to the observations for mutants Δ *griC* and

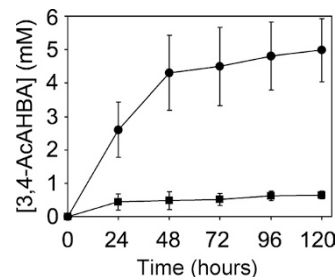


Fig. 4 Bioconversion of 3,4-AHBA to 3,4-AcAHBA by *S. griseus* cells to estimate the membrane permeability for 3,4-AHBA.

Mutant *griG::Km^f [pIJ702-*griG*]* (●) and mutant *griG::Km^f [pIJ702-*melC1'*]* (■) were incubated at 26.5°C in SMM-2.5 supplemented with 10 mM 3,4-AHBA. The concentration of 3,4-AcAHBA in the culture broth was determined by HPLC. Values are expressed as the mean ± standard deviation obtained from three independent experiments.

Δ *griD*, mutant *griG::Km^f* produced grixazone as the wild-type strain (data not shown), indicating that GriG is not essential for grixazone biosynthesis. However, there is a possibility that GriG have a role in the uptake of 3,4-AHBA excreted and/or leaked from the cells during grixazone biosynthesis, because GriG is a homologue of aromatic acid/H⁺ symporters. In fact, GriG enhanced apparent membrane permeability of 3,4-AHBA in *S. griseus* cells as described below.

Mutant *griG::Km^f [pIJ702-*griG*]*, which contained the *griG* sequence under the control of the *melC* promoter [11], and a negative control strain, mutant *griG::Km^f [pIJ702-*melC1'*]*, were cultured for 48 hours in SMM-2.5 (containing 2.5 mM KH₂PO₄) and then 3,4-AHBA (final concentration, 10 mM) was added to the culture to analyze the bioconversion of 3,4-AHBA to 3,4-AcAHBA. SMM-2.5 is more suitable for vigorous growth of *S. griseus* cells than SMM-0.25, but suppresses grixazone biosynthesis [4]. As shown in Fig. 4, mutant *griG::Km^f [pIJ702-*griG*]* converted 3,4-AHBA to 3,4-AcAHBA much more efficiently than mutant *griG::Km^f [pIJ702-*melC1'*]*. Because 3,4-AcAHBA is produced from 3,4-AHBA by the cytosolic SgNAT [9] and it was very unlikely that GriG increases the enzyme activity of SgNAT, these results suggested that mutant *griG::Km^f [pIJ702-*griG*]* strain was more efficient in the uptake of 3,4-AHBA (and/or the excretion of 3,4-AcAHBA) than mutant *griG::Km^f [pIJ702-*melC1'*]*.

Reduction of Some Aryl Carboxylates by Recombinant *S. griseus* Cells

We examined the reduction of some aryl carboxylates by *S.*

griseus [pIJ702-*griCDG*] containing the three genes, *griC*, *griD* and *griG*, under the control of the *melC* promoter. Mutant $\Delta natA$, which is deficient in the ability of *N*-acetylation of 3,4-AHBA [9], was adopted as the host for the bioconversion. In the bioconversion in which an aryl carboxylic acid was added at a final concentration of 10 mM, the most efficient substrate was 3,4-AHBA. 3,4-AHBA added was converted to 3,4-AHBAL (8.0 ± 0.5 mM; data represent the mean \pm standard deviation, obtained from three independent experiments) and 3-acetylamino-4-hydroxybenzaldehyde (3,4-AcAHBAL; 1.5 ± 0.2 mM). 3,4-AcAHBA was not detected. Because 3,4-AcAHBAL was not produced from exogenous 3,4-AcAHBA as described below, it is likely that an acetyltransferase(s) other than SgNAT is responsible for the *N*-acetylation of 3,4-AHBAL. Protocatechuic acid and 4-hydroxybenzoic acid were also converted to protocatechualdehyde (4.5 ± 0.2 mM) and 4-hydroxy-benzaldehyde (1.8 ± 0.4 mM), respectively. In addition, this system converted vanillic acid to vanillin (8.2 ± 0.3 mM), along with the negligible production of vanillyl alcohol (0.05 ± 0.01 mM), which is probably produced by further reduction of vanillin. All of these aryl carboxylates contain a hydroxy group at the *para* position of the carboxyl group. 3,4-AcAHBA, 4-amino-3-hydroxybenzoic acid, benzoic acid, salicylic acid, and 5-aminosalicylic acid were hardly reduced. The bioconversion of some aryl carboxylates described above was not observed in the negative control strain harboring pIJ702-*griG*. It should be mentioned that vanillin, the characteristic aroma component in vanilla, is one of the most important aromatic flavors used widely; a great market demand for natural flavors has brought about a growing interest to produce natural vanillin from natural sources by microbial transformation [20]. This system may have a potential to be employed in production of natural vanillin by microbes, as is suggested for the *Nocardia* CAR system [13].

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References

- Horinouchi S. Mining and polishing of the treasure trove in the bacterial genus *Streptomyces*. *Biosci Biotechnol Biochem* 71: 283–299 (2007)
- Axcel Z, Sabine B-M, Susanne G, Hartmut V, Gerhard S. Preparation of phenoxazinone derivatives by fermentation with *Streptomyces* DSM 3813 and their use as parasiticides. European Patent EP260486 (1988)
- Ohnishi Y, Furusho Y, Higashi T, Chun H-K, Furihata K, Sakuda S, Horinouchi S. Structures of grixazone A and B, A-factor-dependent yellow pigments produced under phosphate depletion by *Streptomyces griseus*. *J Antibiot* 57: 218–223 (2004)
- Higashi T, Iwasaki Y, Ohnishi Y, Horinouchi S. A-factor and phosphate-depletion signals are transmitted to the grixazone biosynthesis genes via the pathway-specific transcriptional activator GriR. *J Bacteriol* 189: 3515–3524 (2007)
- Suzuki H, Ohnishi Y, Furusho Y, Sakuda S, Horinouchi S. Novel benzene ring biosynthesis from C₃ and C₄ primary metabolites by two enzymes. *J Biol Chem* 281: 36944–36951 (2006)
- Suzuki H, Furusho Y, Higashi T, Ohnishi Y, Horinouchi S. A novel *o*-aminophenol oxidase responsible for formation of the phenoxazinone chromophore of grixazone. *J Biol Chem* 281: 824–833 (2006)
- Giddens SR, Feng Y, Mahanty HK. Characterization of a novel phenazine antibiotic gene cluster in *Erwinia herbicola* Eh1087. *Mol Microbiol* 45: 769–783 (2002)
- Núñez LE, Méndez C, Braña AF, Blanco G, Salas JA. The biosynthetic gene cluster for the β -lactam carbapenem thienamycin in *Streptomyces cattleya*. *Chem Biol* 10: 301–311 (2003)
- Suzuki H, Ohnishi Y, Horinouchi S. Arylamine *N*-acetyltransferase responsible for acetylation of 2-aminophenols in *Streptomyces griseus*. *J Bacteriol* 189: 2155–2159 (2007)
- Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate DJ, Smith CP, Ward JM, Schrepf H. Genetic manipulation of *Streptomyces*: a laboratory manual. The John Innes Foundation, Norwich, United Kingdom (1985)
- Katz E, Thompson CJ, Hopwood DA. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. *J Gen Microbiol* 129: 2703–2714 (1983)
- He A, Li T, Daniels L, Fotheringham I, Rosazza JPN. *Nocardia* sp. carboxylic acid reductase: cloning, expression, and characterization of a new aldehyde oxidoreductase family. *Appl Environ Microbiol* 70: 1874–1881 (2004)
- Venkatasubramanian P, Daniels L, Rosazza JPN. Reduction of carboxylic acids by *Nocardia* aldehyde oxidoreductase requires a phosphopantetheinylated enzyme. *J Biol Chem* 282: 478–485 (2007)
- Bentley SD, Chater KF, Cerdeño-Tárraga A-M, Challis GL, Thomson NR, James KD, Harris DE, Quail MA, Kieser H, Harper D, Bateman A, Brown S, Chandra G, Chen CW, Collins M, Cronin A, Fraser A, Goble A, Hidalgo J, Hornsby T, Howarth S, Huang C-H, Kieser T, Larke L, Murphy L, Oliver K, O'Neil S, Rabbinowitsch E, Rajandream M-A, Rutherford K, Rutter S, Seeger K, Saunders D, Sharp S,

- Squares R, Squares S, Taylor K, Warren T, Wietzorrek A, Woodward J, Barrell BG, Parkhill J, Hopwood DA. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* 417: 141–147 (2002)
15. Li L, Bannantine JP, Zhang Q, Amonsin A, May BJ, Alt D, Banerji N, Kanjilal S, Kapur V. The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. *Proc Natl Acad Sci USA* 102: 12344–12349 (2005)
 16. Ueda K, Yamashita A, Ishikawa J, Shimada M, Watsuji T, Morimura K, Ikeda H, Hattori M, Beppu T. Genome sequence of *Symbiobacterium thermophilum*, an uncultivable bacterium that depends on microbial commensalism. *Nucleic Acids Res* 32: 4937–4944 (2004)
 17. Duchaud E, Rusniok C, Frangeul L, Buchrieser C, Givaudan A, Taurit S, Bocs S, Boursaux-Eude C, Chandler M, Charles J-F, Dassa E, Deroose R, Derzelle S, Freyssinet G, Gaudriault S, Médigue C, Lanois A, Powell K, Siguier P, Vincent R, Wingate V, Zouine M, Glaser P, Boemare N, Danchin A, Kunst F. The genome sequence of the entomopathogenic bacterium *Photorhabdus luminescens*. *Nat Biotechnol* 21: 1307–1313 (2003)
 18. Meighem EA. Bacterial bioluminescence: organization, regulation, and application of the *lux* genes. *FASEB J* 7: 1016–1022 (1993)
 19. Pao SS, Paulsen IT, Saier Jr MH. Major facilitator superfamily. *Microbiol Mol Biol Rev* 62: 1–34 (1998)
 20. Priefert H, Rabenhorst J, Steinbüchel A. Biotechnological production of vanillin. *Appl Microbiol Biotechnol* 56: 296–314 (2001)