Identification of the Phenalamide Biosynthetic Gene Cluster in
Myxococcus stipitatus DSM 14675

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Introduction

Myxobacteria, rod-shaped gram-negative soil bacteria, produce diverse bioactive secondary metabolites [15, 21]. The majority of bioactive secondary metabolites isolated from myxobacteria are polyketides (PKs), non-ribosomal peptides (NRPs), and hybrids of polyketide and non-ribosomal peptide (PK-NRPs) [21]. PKs and NRPs are biosynthesized by polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), respectively [22]. PKSs and NRPSs are multidomain enzymes or enzyme complexes whose genes are commonly clustered on the chromosome [22].

Phenalamide, also known as stipiamide, is a hybrid of PK-NRP isolated from Myxococcus stipitatus, a myxobacterium [1, 10, 19]. This compound was at first isolated as a substance that reverses P-glycoprotein-mediated multidrug resistance, a condition common to many cancer cell lines [10]. Later, the inhibitory activity of phenalamide A1 against HIV-1 replication in cell cultures was discovered [19]. Phenalamide A2 was reported to inhibit respiratory complex I [6]. Phenalamides are produced by most M. stipitatus strains, whereas strains of other Myxococcus species such as M. xanthus and M. fulvus do not, and thus production of phenalamide is a unique characteristic of M. stipitatus [11]. However, it is unknown whether all the strains of M. stipitatus produce identical derivatives of phenalamide or not.

The chemical structure of phenalamides is similar to that of myxalamids [7] isolated from Myxococcus xanthus and Stigmatella aurantiaca, except that they utilize a molecule carrying a benzene ring, instead of short branched-chain carboxylic acids, as a starter molecule (Fig. 1) [19]. Myxalamids also block the respiratory chain at the site of complex I [7]. Five derivatives of phenalamides, A1, A2, A3, B, and C, were isolated from M. stipitatus strains [21]. However, the natural biosynthetic genes have not been discovered until now. In this paper, we report the

Phenalamide is a bioactive secondary metabolite produced by Myxococcus stipitatus. We identified a 56 kb phenalamide biosynthetic gene cluster from M. stipitatus DSM 14675 by genomic sequence analysis and mutational analysis. The cluster is comprised of 12 genes (MYSTI_04318-MYSTI_04329) encoding three pyruvate dehydrogenase subunits, eight polyketide synthase modules, a non-ribosomal peptide synthase module, a hypothetical protein, and a putative flavin adenine dinucleotide-binding protein. Disruption of the MYSTI_04324 or MYSTI_04325 genes by plasmid insertion resulted in a defect in phenalamide production. The organization of the phenalamide biosynthetic modules encoded by the fifth to tenth genes (MYSTI_04320-MYSTI_04325) was very similar to that of the myxalamid biosynthetic gene cluster from Stigmatella aurantiaca Sg a15, as expected from similar backbone structures of the two substances. However, the loading module and the first extension module of the phenalamide synthase encoded by the first to fourth genes (MYSTI_04326-MYSTI_04329) were found only in the phenalamide biosynthetic gene cluster from M. stipitatus DSM 14675.

Keywords: Myxobacteria, Myxococcus stipitatus, secondary metabolite, phenalamide, biosynthetic gene
identification of the phenalamide biosynthetic gene cluster in M. stipitatus DSM 14675.

Materials and Methods

Strains and Media

M. stipitatus DSM 14675 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). M. stipitatus KYC592 and KYC593 were constructed by inserting plasmids pSH105 and pSH109 into the chromosome of strain DSM 14675 in this study. CYE medium [16] was used for the vegetative growth of the strains, and CYS broth [17] was used for phenalamide production.

Sequence Analysis

Secondary metabolic genes were identified using the antiSMASH (antibiotics & Secondary Metabolite Analysis SHell) program [14, 20]. DNA and amino acid sequences were analyzed using the BLAST [2] and CD-Search [13] programs.

Plasmid and Strain Construction

pSH105 was constructed by inserting an internal DNA fragment (478 bp) of MYSTI_04324 into the pCR2.1 plasmid (Invitrogen, USA). The internal DNA fragment was PCR-amplified using two oligonucleotides, 5'-GATTCCTCCACTCCTGCACC-3' and 5'-GAACACGAACACGACCTTGC-3', as primers and the genomic DNA of M. stipitatus DSM 14675 as a DNA template. pSH109 was constructed by inserting an internal DNA fragment (493bp) of MYSTI_04325 into the pCR2.1 plasmid. The internal DNA fragment was PCR-amplified using two oligonucleotides, 5'-GTCCTGCTGAAGAACTCGCT-3' and 5'-CGGTCAAGTCGTAGGTCTCG-3', as primers and the genomic DNA of M. stipitatus DSM 14675 as a DNA template.

Plasmid insertion mutants were constructed by the methods described previously [4]. Plasmids were introduced into the wild-type strain DSM 14675 by electroporation, and kanamycin-resistant transformants allowed only cells carrying an integrated plasmid on the chromosome to grow. Insertion of the plasmids in designated locations was confirmed by polymerase chain reaction using a set of oligonucleotides as primers: one binds to the pCR2.1 vector DNA and the other binds to the chromosomal DNA near the insertion site. Two oligonucleotides, 5'-GGCGATAAATTTACACAGG-3' (M13R) and 5'-GCCACGTCACCCTCACCCA-3', were used to confirm insertion of pSH105 in the designated location, and M13R and another oligonucleotide, 5'-GAAGTTGAGTGAGTTGAGGA-3', were used to detect the insertion of pSH109.

Preparation of Culture Extract

Bacterial cells were cultured in CYS liquid medium with 2% Amberlite XAD-16 resin for 7 days at 32°C. Harvested cells were treated with acetone to disrupt the cells and extracted with ethyl acetate. After the ethyl acetate was evaporated from the extract, the resulting residue was dissolved in 80% methanol.

LC-MS/MS Analysis

Liquid chromatography mass spectrometry (LC-MS) was performed using an Acela UHPLC (Thermo Scientific, USA) equipped with a Waters BEH C18 column (2.1 x 150 mm, 1.7 µm) and an LTQ-Orbitrap XL high resolution mass analyzer (Thermo Scientific). Mobile phase A was water and mobile phase B was acetonitrile – both contained 0.1% formic acid. The gradient elution at a flow rate of 0.4 ml/min was performed as follows: 0–20 min of 5–70% B (linear gradient), 20–24 min of 70–100% B (linear gradient), and 24–27 min of 100% B (isocratic). The mass spectra were obtained in the range m/z 100–1,500 using a HESI ionization source.

Results and Discussion

Analysis of the Secondary Metabolic Genes of M. stipitatus DSM 14675

Phenalamide is a fluorescent substance produced by most M. stipitatus strains [11]. M. stipitatus DSM 14675, the neotype strain of M. stipitatus, also produces fluorescent substances. Thus, it was expected that the DSM 14675 strain would produce phenalamides. Phenalamide is a polyketide/non-ribosomal peptide hybrid with a benzene ring at one end and alanine at the other end [10, 19]. The chemical structure of phenalamides is similar to that of myxalamids whose biosynthetic genes are known [3, 18]. Therefore, we predicted that the phenalamide biosynthetic gene cluster would consist of genes encoding several PKS modules and a gene encoding a NRPS module, similar to the myxalamid biosynthetic gene cluster. The genomic DNA sequence of M. stipitatus DSM 14675 has been reported [9].

To identify the phenalamide biosynthetic genes from the DSM 14675 strain, the genomic DNA sequence of the strain
(Accession No. CP004025) was analyzed using the antiSMASH program [14, 20], which identifies secondary metabolic genes in DNA sequences. A total of 26 clusters were detected to encode PKS and/or NRPS. Among them, genes in the genomic region 5,512,656–5,568,890bp were predicted to produce substances similar to phenalamides. This region contained 12 genes (MYSTI_04318 to MYSTI_04329) transcribed in the same direction. The transcription direction was the reverse of the order of gene numbers. Since 10 genes from MYSTI_04329 to MYSTI_04320 were either translationally coupled or spaced with less than 17 bp between adjacent genes, we proposed that these genes might constitute an operon. The fourth to ninth genes were predicted to encode PKS modules. The tenth gene was predicted to encode a NRPS module, which is specific to alanine. Organization of the PKS/NRPS modules encoded by the fifth to tenth genes (MYSTI_04320-MYSTI_04325) were found to be similar to that of the myxalamid biosynthetic gene cluster from *S. aurantiaca* Sg a15.

To test whether the MYSTI_04318-04329 region contained the hypothesized phenalamide biosynthetic genes, we created two mutants, KYC592 and KYC593, in which MYSTI_04324 and MYSTI_04325, respectively, were inactivated by plasmid insertion (Fig. 2A). The pSH105 plasmid carrying an internal DNA fragment of MYSTI_04324 was used to create KYC592, and the pSH109 plasmid carrying

![Figure 2. Alignment of operons.](image)

(A) Putative phenalamide biosynthetic locus of *Myxococcus stipitatus* DSM 14675. Arrows show the locations of plasmid insertion mutations that disrupt phenalamide biosynthesis in strains KYC592 and KYC593. (B) Myxalamid biosynthetic locus of *Stigmatella aurantiaca* Sg a15 [18].

![Figure 3. Lack of fluorescent substance production in *Myxococcus stipitatus* after plasmid insertion mutations.](image)

(A, D) DSM 14675 parent strain. (B, E) KYC592 strain with gene disruption. (C, F) KYC593 strain with gene disruption. (A–C) Visible light. (D–F) UV light, which stimulates fluorescence in the parent strain.
an internal DNA fragment of MYSTI_04325 was used to create KYC593.

Colonies of *M. stipitatus* DSM 14675 display yellow fluorescence under ultraviolet light due to the production of phenalamides. Thus, it was expected that if the MYSTI_04318-04329 region was involved in phenalamide biosynthesis, the two insertion mutants, KYC592 and KYC593, would lose fluorescence. When KYC592 and KYC593 were grown on CYE plates for 3 days and exposed to ultraviolet light, their colonies did not show any fluorescence (Figs. 3E–3F). Under the same condition, colonies of wild-type strain DSM 14675 displayed bright yellow fluorescence (Fig. 3D). These were the first experimental data showing that the MYSTI_04318-04329 region was involved in phenalamide biosynthesis. The mutants did not show any differences from the wild type in growth rate, motility, and fruiting body formation, only differing in the color of the colonies.

**Lack of Phenalamide Production after Gene Disruptions**

To confirm that the fluorescent substance defective in the plasmid insertion mutants was phenalamide, culture extracts of DSM 14675 and KYC592 were prepared and analyzed with UHPLC-MS. The culture extract of DSM 14675 showed several peaks near 22 min (Fig. 4A) and those peaks were not found with the culture extract of KYC592 (Fig. 4A). When these peaks of DSM 14675 culture extract were analyzed with a high-resolution mass analyzer, the 22.30 min peak displayed a major signal of 474.3353 m/z ([M+H]+) (Fig. 4B). The 22.53 min peak displayed a major signal with 492.3460 m/z ([M+H]+) (Fig. 4B). It was predicted that the substance with 492.3460 m/z was $C_{32}H_{46}O_3N$, and the substance with 474.3353 m/z was a dehydrated form of $C_{32}H_{46}O_3N$. The molecular formula of phenalamides A1, A2, and A3 is $C_{32}H_{46}O_3N$ and addition of a proton to it yields $C_{32}H_{47}O_3N$. Thus, this result confirmed that the peaks missing from the KYC592 culture extract were phenalamides. Therefore, it was concluded that the MYSTI_04318-04329 region of *M. stipitatus* DSM 14675 was the phenalamide biosynthetic gene cluster.

**Analysis of the Phenalamide Biosynthetic Gene Cluster**

The first three genes (MYSTI_04329-04327) in the phenalamide biosynthetic gene cluster were predicted to encode pyruvate dehydrogenase subunits (Fig. 2, Table 1), which would be involved in acetyl-CoA generation. Phenalamide is expected to use a substance carrying a benzene ring as a starter molecule. Therefore, it is possible that the protein complex encoded by the first three genes uses phenylpyruvate as a substrate and generates phenylacetyl-CoA, which then becomes the starter molecule for phenalamide synthesis. The third gene (MYSTI_04327) encodes an acyl carrier protein (ACP) domain at the C-terminal end, which would be considered a loading module.

The fourth gene (MYSTI_04326) was predicted by the antiSMASH program to encode polyketide synthetase modules carrying ketosynthase (KS), methylmalonate specific acyl transferase (AT), dehydrase (DH), enoyl reductase (ER), keto reductase (KR), and ACP domains (Table 1). Each of the fifth, sixth, and seventh genes (MYSTI_04325, MYSTI_04324, and MYSTI_04323) was predicted to encode a polyketide synthetase module carrying KS, methylmalonate-specific AT, DH, KR, and ACP domains (Table 1). The

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**Fig. 4.** UHPLC-MS analysis of *Myxococcus stipitatus* culture extracts. (A) UHPLC chromatograms (total scan PDA) of the culture extracts of DSM 14675 parent strain and KYC592 with gene disruption. (B) Mass spectra of the substances at retention times 22.30 min and 22.53 min, which disappeared in the culture extract of *M. stipitatus* KYC592.
The eighth gene (MYSTI_04322) was predicted to encode three polyketide synthetase modules carrying KS, malonate-specific AT, DH, ER, KR, and ACP domains (Table 1). The ninth gene (MYSTI_04321) was predicted to encode a polyketide synthetase module carrying KS, methylmalonate-specific AT, DH, ER, KR, and ACP domains (Table 1). However, the ER domain of the ninth gene appeared to be defective since a region of about 33 amino acid residues, which were conserved in ERs of other PKSs, was missing (Fig. 5). The tenth gene (MYSTI_04320) was predicted to encode a non-ribosomal peptide synthetase module specific to alanine (Table 1). The eleventh gene was predicted to encode a hypothetical protein, and the twelfth gene a flavin adenine dinucleotide-binding protein (Table 1).

The organization of phenalamide biosynthetic modules in M. stipitatus DSM 14675 is similar to that of myxalamid biosynthetic modules from S. aurantiaca Sg a15, with a few discrepancies (Table 1). First, they have different loading modules. The phenalamide biosynthetic enzyme complex appears to load phenylacetyl-CoA. In contrast, the myxalamid biosynthetic enzyme complex loads acetyl-CoA or isobutyl-CoA. Second, the phenalamide biosynthetic enzyme complex has one more extension unit (MYSTI_04326) than the myxalamid biosynthetic enzyme complex. Third, module 8 of the phenalamide biosynthetic enzyme complex is encoded by a gene (MYSTI_04321) in M. stipitatus, but the same module of the myxalamid biosynthetic enzyme complex is encoded by two genes, mxaB1 and mxaB2.

We propose a model for phenalamide biosynthesis in M. stipitatus DSM 14675 based on the organization of modules and the chemical structure of phenalamide (Fig. 6). The final product predicted based on the sequence analysis was identical to phenalamide A1, A2, and A3 in molecular weight and backbone structure. However, the

Table 1. Comparison of the putative phenalamide biosynthetic genes from Myxococcus stipitatus DSM 14675 with the myxalamid biosynthetic genes from Stigmatella aurantiaca Sg a15.

<table>
<thead>
<tr>
<th>Phenalamide biosynthetic genes from Myxococcus stipitatus DSM 14675</th>
<th>Myxalamid biosynthetic genes from Stigmatella aurantiaca Sg a15</th>
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ACP, acyl carrier protein; KS, ketosynthase; AT, acyl transferase; DH, dehydrase; ER, enoyl reductase; KR, keto reductase; C, condensation domain; A(ala), alanine adenylase; PCP, peptidyl carrier protein; TD, terminal reductase domain; mal, malonyl CoA; mmal, methylmalonyl CoA; ala, alanine. Domains of the myxalamid biosynthetic genes not found in the corresponding phenalamide biosynthetic gene are underlined. Similarity: similarity to the corresponding proteins of the myxalamid biosynthetic enzymes.
Fig. 5. Alignment of enoyl reductase domains.
The enoyl reductase (ER) domains of MYSTI_04321 and MYSTI_04326 were aligned with the ER domains of the myxalamid synthase from *Stigmatella aurantiaca* (MxaB1_ER) [18] and the erythronolide synthase from *Saccharopolyspora erythraea* (EryA_ER) [5]. Identical amino acid residues are inverted and similar residues are shaded. Asterisk indicates putative NAD(P)-binding sites [12].

Fig. 6. Model for phenalamide biosynthesis in *Myxococcus stipitatus* DSM 14675.

- PKS domains;  - NRPS domains. Asterisk indicates a presumably inactive domain.
detail structure of the predicted product was different from the known phenalamides. Phenalamide A1, A2, and A3 have a hydroxyl group at C-13, but the predicted product had a hydroxyl group at C-7. This was caused by the presence of a DH domain at module 4 and the absence of a DH domain at module 6. The DH domain of module 4 had all the conserved residues for dehydrogenase activity and there was no trace of the DH sequence in module 6. Thus, it was natural to accept that the DH domain of module 4 was active and there was no DH activity in module 6, although no phenalamide derivative with the predicted structure was found until now. It is possible that structures of phenalamides vary among different strains of M. stipitatus, and DSM 14675 strain produces phenalamides with a hydroxyl group at C-7, unlike other M. stipitatus strains from which phenalamides have been identified.

Acknowledgments

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References