Biosynthesis of 2'-O-Methylmyxalamide D in the Myxobacterium Cystobacter fuscus: a Polyketide Synthase-Nonribosomal Peptide Synthetase System for the Myxalamide D Skeleton and a Methyltransferase for the Final O-Methylation

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The biosynthetic gene cluster for the polyene antifungal antibiotic, 2'-O-methylmyxalamide D, was cloned from myxobacterium Cystobacter fuscus AJ-13278. A sequence analysis of the 12.8-kb region in the gene cluster revealed the presence of two type I polyketide synthase genes, mmxB and mmxC. The involvement of these two genes in the biosynthesis of 2'-O-methylmyxalamide D was confirmed by a gene disruption experiments. In addition, an S-adenosylmethionine-dependent methyltransferase gene (mmxM) was found downstream of the gene cluster and demonstrated, by a gene disruption analysis, to be responsible for converting the known unmethylated precursor, myxalamide D, into 2'-O-methylmyxalamide D.

Key words: biosynthetic gene cluster; myxobacterium; antifungal agent; polyketide synthase; methyltransferase

Myxobacteria have been shown to be outstanding producers of novel bioactive secondary metabolites. Some of these secondary metabolites, including antifungal antibiotics, and antibacterial and anticancer agents, are polyketides, peptides, or their hybrids, which are biosynthesized by polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS). PKS and NRPS possess modular organization, and each module catalyzes one chain-elongation step in their biosynthetic process. Polyketides and nonribosomal peptides are widely used as a source of medicinal agents, and their biosynthetic multienzymes, PKS and NRPS, have led to the emergence of a new horizon for the engineered biosynthesis of unnatural bioactive products through the use of the molecular genetic methodology.

We have previously isolated the antifungal antibiotic, 2'-O-methylmyxalamide D (Fig. 1), from the myxobacterium, Cystobacter fuscus strain AJ-13278. The chemical structure of 2'-O-methylmyxalamide D suggests that it is biosynthesized by PKS/NRPS enzymes. The biosynthetic gene (mxa) cluster for myxalamides (Fig. 1), one of which is a precursor of 2'-O-methylmyxalamide D, has recently been isolated from another myxobacterium, Stigmatella aurantiaca strain Sga15. The mxa gene cluster consists of six PKS genes and an NRPS gene for encoding a hybrid PKS/NRPS system. A structural comparison between 2'-O-methylmyxalamide D and myxalamides A-D is shown in Fig. 1.

Fig. 1. Chemical Structures of 2'-O-Methylmyxalamide D Isolated from C. fuscus and of the Myxalamides from S. aurantiaca.
amidase D indicates that 2′-O-methylmyxalamide D should be biosynthesized not only by a PKS/NRPS system similar to Mxa, but also by an additional gene encoding a methyltransferase for the final methylation of myxalamide D into 2′-O-methylmyxalamide D. We describe in this paper the cloning of the gene cluster for 2′-O-methylmyxalamide D biosynthesis in C. fuscus, the sequence analysis of the gene encoding the myxalamide D 2′-O-methylating enzyme, and the partial sequence analysis of an mxa-like gene cluster involved in the biosynthesis.

Materials and Methods

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. The Escherichia coli strains were cultured in a Luria-Bertani (LB) medium at 37°C. The C. fuscus strains were cultured in a Casitone medium (2% Difco Casitone, 0.1% MgSO₄·7H₂O, and 10 mm HEPES at pH 7.2 with NaOH) or a production medium (1% Difco Casitone, 0.5% dried yeast, 0.2% malt extract, 0.1% yeast extract, 0.1% MgSO₄·7H₂O, 0.3% Mg₃(PO₄)₂·8H₂O, 1% HEPES, 0.05% agar, and 2% SEPABEADS SP207 resin (Mitsubishi Chemical)) as described previously.9 An antibiotic was added, if necessary, in the following final concentration: 50μg/ml of kanamycin sulphate (Wako), 12.5μg/ml of chloramphenicol (Wako), or 50μg/ml of ampicillin (Wako).

PCR, sequencing, and general DNA manipulation. Chromosomal DNA of the C. fuscus strains was prepared as described in the literature.10 PCR was performed with a GeneAmp PCR 9700 system (Applied Biosystems), using Ex Taq DNA polymerase (Takara). DNA sequences were determined with a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). All other DNA manipulation procedures were performed according to the standard protocols.11

Screening of a bacterial artificial chromosome (BAC) library for the 2′-O-methylmyxalamide D biosynthetic gene (mmx) cluster. The degenerate primers, KS1Up and KS1D,12 were used for the PCR amplification of DNA encoding β-ketoacyl synthase (KS) from C. fuscus AJ-13278. An approximately 700-bp PCR product was ligated into the pCR2.1-TOPO vector by using the TOPO TA cloning kit (Invitrogen). The transformation of E. coli was performed by following the manipulation procedure in the kit. The KS DNA fragments, KSM4 and KSM6, were used as the probes to screen the previously constructed BAC library by colony hybridization. A Gene Images AlkPhos Direct Labelling and Detection System (Amersham Biosciences) was used for probe labeling and signal detection. Three BAC clones, p4-4G, p4-7C and p7-7B, indicated positive signals against both the probes (Fig. 2A).

Partial sequencing of BAC containing the mmx gene cluster. BAC clone p4-4G was digested with HindIII, and the 17-kb fragment containing both the KSM4 and KSM6 sequences was cloned into HindIII-digested pCC1BAC™ (Epigen). The ligation product was transferred into E. coli EPI300™ (Epigen) to give the transformant, sub4. Plasmid psub4 from sub4 was digested with Clal, and the 7.7-kb and 4.5-kb fragments were subcloned into pBluescript II SK to respectively make psub8 and psub9 (Fig. 2A). Plasmids psub8 and psub9 were further subcloned, and the resulting products were sequenced. Successive downstream DNA (550 bp) was further sequenced by primer walking on psub4 to provide the 12.8-kb region of the mmx gene cluster. The sequence of this region has been deposited in the GenBank database under accession no. DQ190053.

Cloning of a relevant gene encoding a methyltransferase. BAC clone p7-7B was digested with HindIII, and the vector-containing fragment (13 kb) was self-ligated to make psub7. Plasmid psub7 was digested with XhoI, and the resulting fragments were subcloned into XhoI-

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<td>ET12567</td>
<td>Host for preparation of non-methylated plasmids</td>
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Table 1. Bacterial Strains Used in This Study
digested pBluescript II SK. Partial sequencing of the subclones found one (psub44) encoding an enzyme similar to methyltransferases (Fig. 2A). Complete sequencing and a BLAST analysis of psub44 indicated the presence of an 867-bp ORF (mmxM) homologous to the methyltransferase genes. The sequence of mmxM has been deposited in the GenBank database under accession no. DQ190054.

Generation of C. fuscus mutants and HPLC analysis of their secondary metabolites. All experiments were carried out according to the previously described procedures, unless otherwise mentioned.9) The C. fuscus mutants examined in this study are listed in Table 1. To obtain a methyltransferase gene (mmxM) disrupted mutant, a DNA fragment (766 bp) of mmxM was amplified by PCR with the pair of primers, 5'-AGTC-CAGTACACGGCGGAAGGCG-3' and 5'-GTGGC-TACGCGTTGCGGAGAT-3', and cloned into pCR2.1-TOPO by using the TOPO TA cloning kit (Invitrogen). The resulting plasmid, pOME, was transformed into wild-type C. fuscus to give the mutant, MOME. The other two mutants, MKSM4 and MKSM6, were generated by the same procedure with respective plasmids, pKSM4 and pKSM6. The homologous insertion of a DNA fragment in the resulting mutants was confirmed by PCR, as shown in Fig. 3, using MOME and MKSM6 as the examples. The sequences of primers C2–C5 were as described previously.9) C1, 5'-ATGCGGTCCCACT-CCACATGCGGGTTG-3'; C6, 5'-GTCCCGGCAGCG-TGTTCGAGGTTG-3'; C1', 5'-CGGCACTCCCAG-GCAATCAAATAACA-3'; C6', 5'-GCCAGGGTGCTC- GTTGACCTTCTCG-3'. The mutant strains were cultured in a production medium, and their secondary metabolites were extracted with acetone for an HPLC analysis as described previously.9)

Spectroscopic analysis of the products of C. fuscus mutant MOME. The C. fuscus mutant, MOME, was first cultured in the Casitone medium for 3 d, and a 3-ml portion was inoculated into 1-liter of the production medium. After being cultured for 4 d, the cells and the resin were collected by centrifugation and extracted twice with acetone (200 ml each). The combined acetone extracts were concentrated to give an aqueous mixture (10 ml), which was extracted 3 times with EtOAc (10 ml each). The EtOAc extract (321 mg) was chromatographed on silica gel (Hi Flash™, S size, 16 × 60 mm, 6 g, Yamazen), eluting with 3–33% acetone in benzene (a linear gradient over 60 min) at a flow rate of 3 ml/min. The fractions (71.6 mg) eluted with 22–28% acetone in benzene were combined and purified by HPLC (Develosil ODS-UG-5, 20 × 250 mm, 75% aq. MeOH, UV 240 nm) to give myxalamide D (2.8 mg) as

Fig. 2. Map of the Sequenced mmx Genes and Domain Arrangement of the Deduced Proteins. A, Mmx-containing DNA fragments cloned into the BACs and location of the subcloned fragments, mmxC, mmxB and mmxM. The two blocks, KSM4 and KSM6, show the regions amplified by PCR and were used for gene disruption. H, HindIII; H*, HindII in vector. B, Arrangement of a partial mxa gene cluster for myxalamide biosynthesis.9) C, PKS domain arrangement deduced from the sequenced 12.8-kb DNA region.
Results and Discussion

Two KS DNA fragments involved in the biosynthesis of 2'-O-methylmyxalamide D

KS DNA fragments can be obtained by PCR, using primers degenerated on the basis of the conserved amino acid sequences of the KS domains in type I PKS. We have previously obtained a KS fragment involved in the cystothiazole biosynthetic gene (cta) cluster by PCR with two degenerate primers. In this present study, another pair of degenerate primers, KS1Up and KSD1, was used, and six unique KS fragments, named KSM1–KSM6, were obtained. These fragments possessed a homologous sequence to that of the KS domains in the type I PKS gene. Of them, KSM1 was identical to a KS fragment in cta. The deduced amino acid sequence of KSM2 was homologous with the KS domain in StiA (accession number CAD19085, identity/similarity of 64%/78%), which is a PKS in stigmatellin biosynthesis. The deduced amino acid sequences of KSM3 to KSM6 showed high homology with the KS domains in the myxalamide biosynthetic multi-enzyme system, Mxa: KSM3, 90%/94% identity/similarity to the first KS domain in MxaC; KSM4, 82%/87% to the second KS domain in MxaC; KSM5, 92%/96% to the KS domain in MxaE; and KSM6, 91%/96% to the KS domain in MxaB1. The KSM2–KSM6 fragments were cloned into pCR2.1-TOPO, and the resulting plasmids were used for gene disruption. The plasmids containing KSM4 and KSM6 respectively gave the kanamycin-resistant transformants, MKSM4 and MKSM6, while no transformant was obtained with the other plasmids. Homologous integration of the plasmids in MKSM4 and MKSM6 was verified by PCR, the result for MKSM6 being shown in Fig. 3B. An HPLC analysis of the extracts of the mutants indicated that the production of 2'-O-methylmyxalamide D has been completely arrested (Figs. 4B and C), suggesting that the KSM4 and KSM6 fragments were located within the 2'-O-methylmyxalamide D biosynthetic mmx gene cluster.

Isolation of BAC clones containing the mmx gene cluster

To obtain the 2'-O-methylmyxalamide D biosynthetic gene (mmx) cluster, the previously constructed BAC library was screened. DNA fragments KSM4 and KSM6 were used as probes for this screening by colony hybridization, and three positive clones that hybridized to both the probes were selected. The DNA sequences of the insertion termini of these clones indicated the presence of any PKS/NRPS homologous sequence in p7-7B. The presence of a PKS homologous sequence in p4-4G, and the presence of an NRPS homologous sequence in p4-7C (Fig. 2). Since the mmx gene cluster, containing seven genes, is about 50 kb, the p7-7B clone (80 kb) was expected to contain the complete mmx gene cluster in the inserted fragment. Studies on the biosynthetic gene clusters of natural products have widely

\[\text{ESI-TOF-MS: } m/z = 374.2685 [M + H]^+; \text{ calcd. for C}_{23}\text{H}_{36}\text{NO}_3, 374.2690.\]
used a cosmid library rather than a BAC library. However, a BAC library should be advantageous, at least for cloning the complete gene cluster in a single clone, because of its capacity for large insertions. We have previously cloned the complete gene cluster (cta) of cystothiazole biosynthesis from the same BAC library and this time successfully cloned another biosynthetic gene cluster (mmx).

**Partial characterization of the mmx gene cluster**

The 12.8-kb region of the 2'-O-methylmyxalamide D biosynthetic mmx gene cluster was sequenced. The sequence revealed this region to be part of the gene cluster as shown by the subsequent analysis.

The DNA and deduced amino acid sequences were analyzed by FramePlot and BLAST. The sequenced DNA region consists of 12,757 bp with a typical myxobacterial G + C content of 71.9%. Two overlapped open reading frames (ORFs), named mmxC and mmxB, are located in this region. The overlapped sequence is ATGA, in which TGA is used for the upstream ORF (mmxC) as the stop codon, while ATG is used for the downstream ORF (mmxB) as the start codon. These two genes are transcribed in the same direction. The start codon of mmxC and the stop codon of mmxB must be located outside the sequenced 12.8-kb region. The sequenced parts of MmxC and MmxB show high homology to their counterparts, MxaC and MxaB1, of the myxalamide biosynthetic multienzymes (identity/similarity of 78%/85% and 84%/88%, respectively) in spite of the genetic difference involved between the two myxobacteria. The deduced amino acid sequences of MmxC and MmxB contain several domains typical of PKS (Fig. 2C). The sequenced part of MmxC contains two KS domains, two acyl transferase (AT) domains, two β-ketoacyl reductase (KR) domains, one β-hydroxylacyl-ACP dehydratase (DH) domain, and two acyl carrier protein (ACP) domains. These domains are arranged as shown in Fig. 2C to form two PKS modules responsible for diketide formation (C-3 to C-6, carbon numbers of 2'-O-methylmyxalamide D) in the 2'-O-methylmyxalamide D biosynthesis, based on a comparison of the gene arrangements and chemical structures between 2'-O-methylmyxalamide D and myxalamide D. The sequenced part of MmxB contained one KS and one AT domain, which both seem to be responsible for the next monoketide formation (C-1 to C-2).

In the biosynthesis of myxalamides (the mxa gene cluster), PKSs construct the polyketide carbon chain, and finally an NRPS catalyzes the condensation of alanine to the carboxyl terminal to form an amide bond. An approximately 500-bp sequence homologous to the NRPS gene of the mxa cluster was found at the right-hand terminus of the p4-7C insertion (Fig. 2A), which is 4 kb downstream from the sequenced part of mmxB. In the mxa gene cluster, the NRPS gene (mxaA) is located 3.7 kb downstream from mxaB1 (Fig. 2B). These findings and structural similarity suggest that 2'-O-methylmyxalamide D should be biosynthesized by a PKS-NRPS hybrid gene cluster similar to that of the myxalamides.

**Cloning and analysis of a methyltransferase gene**

The sequenced part of the mmx gene cluster showed high homology to its counterpart of the mxa gene cluster from *S. aurantiaca*, as already mentioned. The structural difference between their metabolites (Fig. 1) and the sequence result (Fig. 2) suggests that the arrangement of the mmx genes is similar to that of the mxa genes, and that an additional enzyme gene, probably a methyltransferase gene involved in the 2'-O-methylmyxalamide biosynthesis, is present, being closely linked to the mmx cluster.

The p7-7B BAC clone (Fig. 2A) was subcloned, and one subclone, psub44, was found to contain a methyltransferase-homologous gene (Fig. 2A). This gene (867 bp), named mmmM, encodes a 288-aa enzyme and seems to be responsible for the formation of the 2'-O-methyl ether group. A homology search of MmmM by BLASTP in GenBank gave related proteins, some of which were identified as S-adenosylmethionine-dependent methyltransferases (SAM-MT). MmmM shows the highest homology to Mit, a methyltransferase involved in the biosynthesis of the antitumor antibiotic, mitomy-
cin C, from Streptomyces lavendulae (47% identify, 32% similarity).\(^{16}\) The SAM-MT enzymes, generally only weakly similar to one another, generally contain a highly conserved sequence known as Kagan and Clarke binding motifs I–III.\(^{17}\) MmxM indeed possesses the corresponding sequence: motif I, QPGQRALDVGC-

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586-bp in experiment was performed by homologous recombination. The internal fragment of 766-bp in mmxM was amplified by PCR and cloned into pCR2.1-TOPO. The resulting plasmid was transformed into C. fuscus, and the transformant, MOME, was obtained. Gene disruption in MOME was confirmed by PCR (Fig. 3C). An HPLC analysis of the products from mutant MOME indicated that 2'-O-methylmyxalamide D had disappeared, and trace peaks of shorter retention time (\(~8.4\) min) emerged in a similar intensity to that of 2'-O-methylmyxalamide D (Fig. 4D). The peak fractions were collected and analyzed by \(^1\)H-NMR and MS, indicating that the major product was identical to the known antibiotic, myxalamide D,\(^{18}\) the unmethylated form of 2'-O-methylmyxalamide D. The adjacent minor peak, which indicated the same UV and MS spectra and a similar \(^1\)H-NMR spectrum, was deduced to be an unidentified cis-trans isomer of myxalamide D based on the observation of easy cis-trans interconversion under light. The time-dependent production of myxalamide-related compounds of the wild type and the MOME mutant of C. fuscus were analyzed daily by HPLC, suggesting that more than 90% of myxalamide D produced by the biosynthetic multienzyme, Mmx, was immediately methylated into 2'-O-methylmyxalamide D by MmxM (data not shown).

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References


15) Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and

