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Cloning, Sequencing, and Characterization of the Pradimicin Biosynthetic Gene Cluster of *Actinomadura hibisca* P157-2

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Abstract Pradimicins are potent antifungal antibiotics having an unusual dihydrobenzo[α]naphthacenequinone aglycone substituted with D-alanine and sugars. Pradimicins are polyketide antibiotics produced by *Actinomadura hibisca* P157-2. The gene cluster involved in the biosynthesis of pradimicins was cloned and sequenced. The pradimicin gene cluster was localized to a 39-kb DNA segment and its involvement in the biosynthesis of pradimicin was proven by gene inactivation of *prmA* and *prmB* (ketosynthases α and β). The pradimicin gene cluster consists of 28 open reading frames (ORFs), encoding a type II polyketide synthase (PKS), the enzymes involved in sugar biosynthesis and tailoring enzymes as well as two resistance proteins. The deduced proteins showed strong similarities to the previously validated gene clusters of angucyclic polyketides such as rubromycin, griseorhodin, and fredericamycin. From the pradimicin gene cluster, *prmP3* encoding a component of the acetyl-CoA carboxylase complex was disrupted. The production levels of pradimicins of the resulting mutants decreased to 62% of the level produced by the wild-type strain, which indicate that the acetyl-CoA carboxylase gene would have a significant role in the production of pradimicins through supplying the extender unit precursor, malonyl-CoA.

Keywords: *Actinomadura hibisca*, pradimicin, type II polyketide synthase, polyketide biosynthesis

Polyketides form a large group of biologically active natural products that are synthesized by polyketide synthase (PKS) complexes via a sequence of Claisen condensations of acyl-CoA precursors [10]. Aromatic polyketides such as daunorubicin, oxytetracycline, tetracenomycin, and fredericamycin are synthesized by bacterial aromatic polyketide synthases, also termed type II PKSs [10]. Type II PKSs minimally consist of ketoacyl synthase α (KS_α), ketoacyl synthase β (KS_β) (also known as chain-length factor), and an acyl carrier protein [34]. The enzyme complexes catalyze the biosynthesis of the polyketide backbone, which is generated by condensation of an acetyl starter unit and multiple malonyl-CoA extender units. The polyketide backbone is later regiospecifically folded and cyclized by aromatase and cyclase enzymes to form the characteristic linear, angular, or discoid aromatic ring structures [11]. The structures are further modified by oxidation, reduction, methylation, and glycosylation steps to yield the final polyketide antibiotics. The aforementioned synthetic steps provide a wide array of structural diversity to the polyketides.

Over the past decade, the gene clusters encoding many polyketides have been cloned, sequenced [14], and identified, including the angucyclic polyketides jadomycin [7], rubromycin [22], landomycin [35], fredericamycin [34], and oviedomycin [19]. The angucycline group of antibiotics is a family of natural products of the type II polyketides. The angucycline antibiotics share many features, including a tetracyclic ben(a)anthracene aglycone system [24], but the folding pattern of the nascent β-polyketide chains differs from that observed in other well-analyzed type II polyketides such as actinorhodin and tetracencomycin [25]. The isolation of genes responsible for the production of an angucycline can be expected to expand the number of genes currently available for the combinatorial design of hybrid polyketides [9, 17].

Pradimicins are potent antifungal antibiotics produced by *Actinomadura hibisca* P157-2 [32] and *A. verrucosospora* subsp. *neohibisca* [26]. They are angucycline antibiotics having an unusual dihydrobenzo[α]naphthacenequinone aglycon substituted with D-alanine and sugars, thomosamine and xylose [32] (Fig. 1). The biosynthetic origin of pradimicins was established by isotope labeling experiments [12], revealing that pradimicin is a dodecaketide, which is most

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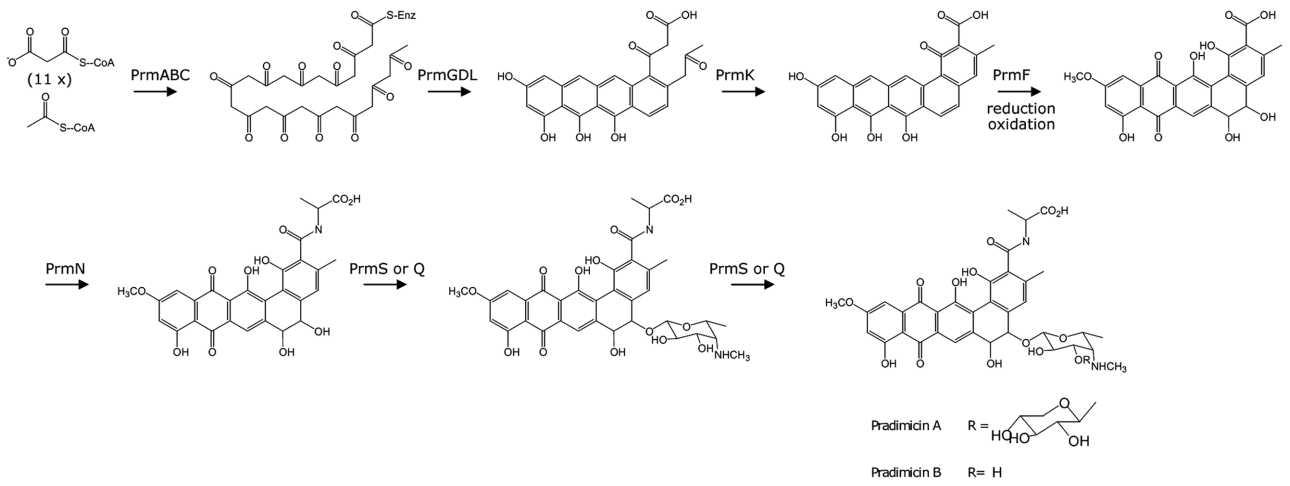

Fig. 1. Proposed biosynthetic pathway for pradimicins.

likely derived from sequential condensations of 11 malonyl-CoA extender units and an acetyl-CoA starter unit (Fig. 1). A previous genetic study claimed to have identified ORFs that are putatively involved in pradimicin A biosynthesis of *A. hibisca* [5]. However, no direct evidence for this supposition was provided. Subsequently, ketosynthase subunits that are essential for pradimicin biosynthesis were identified from *A. verrucosospora* subsp. *neohibisca* [4]. However, to date, no further results regarding the pradimicin biosynthetic gene cluster, other than minimal PKS genes, have been reported.

In the present study, the gene cluster for pradimicin biosynthesis in *A. hibisca* P157-2 has been cloned, sequenced, and confirmed by gene inactivation studies. The sequence analysis has revealed good alignment between the biosynthetic machinery of pradimicin and those of other angucyclic antibiotics such as rubromycin, griseorhodin, and fredericamycin. Ketosynthase genes in the pradimicin biosynthetic gene cluster were disrupted to confirm the involvement of the cloned gene cluster in pradimicin biosynthesis. In addition, the acetyl-CoA carboxylase gene in the pradimicin biosynthetic gene cluster was disrupted to reveal that the gene product has a role in pradimicin production, probably through the supply of the malonyl-CoA precursor.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

All *Escherichia coli* strains used in this study were grown following standard protocols [27]. *E. coli* DH5α was employed for routine gene manipulation. The pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) was used for cloning PCR fragments. The pradimicin producer *A. hibisca* P157-2 (ATCC53557) [32] was maintained on a agar medium composed of 20 g mannitol, 20 g soybean meal, 20 g agar per 1 l of distilled water. For the broth culture, *A. hibisca* was grown at 28°C in ISP2 medium (4 g yeast extract, 10 g malt extract, 4 g dextrose, 1 l distilled water) at pH 7.0. The pradimicin production medium consisted of 30 g glucose, 30 g soybean meal, 5 g pharmamedia, 1 g yeast extract, 3 g $CaCO_3$, and 1 l distilled water. To produce pradimicin, a loop-full of *A. hibisca* wild-type or mutant strains was inoculated into a baffled 1-l flask containing 100 ml of the production medium or ISP2 medium and incubated at 28°C in the dark for 7 days on a rotatory shaker at 170 rpm.

Analysis of Pradimicin Production

The liquid culture (5 l) of *A. hibisca* wild-type and mutant strains was centrifuged at 8,000 rpm for 30 min to remove cells. The supernatant was adjusted to pH 2.0 with 6 N HCl and the supernatant was thoroughly stirred with a mixture of *n*-butanol:methanol (100:1). The organic phase was collected and concentrated *in vacuo* using a rotary evaporator (Büchi, Flawil, Switzerland). The resulting residue was suspended in 5 ml methanol and the crude extracts were chromatographed on a Sephadex LH-20 column (C26/100, GE Healthcare, Uppsala, Sweden). The Sephadex LH-20 column was eluted with methanol at a 0.1 ml/min flow rate. The major red-colored eluant was pooled and analyzed by high performance liquid chromatography (HPLC) using a Gilson HPLC system (Gilson, Middleton, WI, U.S.A.) equipped with a semipreparative C_{18} reversed-phase column (ODS-H80, 1×25 cm, 4 μm, YMC Co., Kyoto, Japan). The column was eluted at a flow rate of 2 ml/min from 20% methanol in water containing 0.1% formic acid to 95% methanol in water containing 0.1% formic acid as a linear gradient over a 50-min uninterrupted interval. The chromatogram was monitored by absorbance at 210 nm. The production rates of pradimicins A and B were quantified

by using standard compounds. Pradimicin standards were prepared from the culture broth of wild-type *A. hibisca* by purification using various chromatographic procedures, as described previously [32]. The purified pradimicins were confirmed by ^{1}H-NMR spectroscopic analyses.

Construction of a Genomic Library and Screening

The total genomic DNA of *A. hibisca* P157-2 (grown on ISP2 medium) was prepared according to the acetyltrimethylammonium bromide procedure for the isolation of genomic DNA [13]. A genomic DNA library was constructed by using pCC2FOS vector as recommended in the manufacturer's protocol (Epicentre Biotechnologies, Madison, WI, U.S.A.). Approximately 1,800 fosmid clones were probed by using the DNA of a digoxigenin-labeled ketosynthase gene, which had been amplified from the *A. hibisca* genomic DNA using a primer set (KS-F: 5'AGC CCA TCA AAG TCC/G ATG A/GTC GG-3'; KS-R: 5'CC GGT GTT C/GAC C/GGC GTA GAA CCA GGC G-3') [34]. All PCR amplifications were performed using the Expanded High Fidelity PCR system according to the manufacturer's instruction (Roche, Basel, Switzerland). Labeling was accomplished using the digoxigenin DNA labeling kit (Roche Diagnosics Corp., Basel, Switzerland). The colony and Southern hybridizations were performed on Hybond N (Amersham Biosciences, Buckinghamshire, U.K.) as recommended by the manufacturer.

Sequencing and Data Analysis

Fosmid DNAs were extracted from *E. coli* by alkaline lysis [27]. Nucleotide sequences were determined by the dideoxy chain termination method, using an automatic sequencer (ABI Prism 3700 DNA sequencer, Applied Biosystems, Foster City, CA, U.S.A.). The DNA sequences were assembled using SeqMan II (DNAStar Inc., Maddison, WI, U.S.A.). The assembled DNA and deduced protein sequences were analyzed using the ORF finder provided by DNAStar Inc., and confirmed by a BLAST database search in the NCBI.

Targeted Disruption of Genes Involved in Pradimicin Biosynthesis

The *prmA* and *prmB* (KS_α and KS_β homologous genes) of the pradimicin biosynthetic gene cluster was disrupted by using the PCR-targeted *Streptomyces* gene replacement method [6]. The *aac(3)IV* apramycin-resistant gene and *oriT* were amplified from the pIJ773 disruption cassette [6] using the primers PRMab-F 5'-CCCCGACGAGCAGGG-GCTCGACGTCGGGGGCCGCACGTGattccggggatccgtc gacc-3' and PRMab-R 5'-GCGGGTTGCCATCTCGTCC-GTCCTCTCGGGTCGTTGTCAtgtaggctggagctgcttc-3' (pIJ773 homologous sequence is in lowercase letters). The resulting PCR-amplified DNA was used to replace the *prmAB* genes in fosmid clone pBC3. The resulting pBC3m was then transferred into *A. hibisca* using established methods [6]. The double crossover mutants were selected on mannitol soya flour agar medium (MS medium: 20 g mannitol, 20 g soy flour, 20 g agar, 1 l distilled water) amended with apramycin (50 μg/ml). The allelic replacement of the *prmAB* genes in the BC3m mutant was confirmed by Southern hybridization analysis, and PCR amplification and sequencing.

For the gene disruption of *orf1*, the apramycin-resistant gene and *oriT* was amplified using the primers PRM1-F 5'-TTCGCCGTTGCAGCCACGCGACGACCGTACCGGG AGATGattccggggatccgtcgacc-3' and PRM1-R 5'-TGACC-ACCGTGCTCAACGC CCTACGAACCCCCGCCGTCA-tgtaggctggagctgcttc-3'. The resulting PCR-amplified DNA was used to replace the *orf1* gene in the fosmid clone pBC4. The resulting pBC4m was then transferred into *A. hibisca* using the established methods [6].

For the gene disruption of *orf30*, the apramycin-resistant gene and *oriT* was amplified using the primers PRM30-F 5'-GGGACCAGGTCCCGGCGGGCCGGTTACGGTG-ACGGCATGattccggggatccgtcgacc-3' and PRM30-R 5'-CTCCCCTGATCCCCGCCGCCCCGGCCAGAAGGC-GGCTCAtgtaggctggagctgcttc-3'. The resulting PCR-amplified DNA was used to replace the *orf30* gene in fosmid clone pBC3. The resulting pBC3-30m was then transferred into *A. hibisca* using the established methods [6].

The gene *prmP3* (acetyl-CoA carboxylase homologous gene) was disrupted using the apramycin-resistant gene and *oriT*, which was amplified using PRMp3-F 5'-GACCG-TGACGACCGACACGACCGGCTCCGCCGCCGAGT Gattccggggatccgtcgacc-3' and PRMp3-R 5'-ATGCCGTG-TCCGGCGCCGCGGGGGATGTCTCGGCCATCAtgta-ggctggagctgcttc-3'. The amplified DNA was used to replace the *prmP3* gene in fosmid clone pBC3. The resulting pBC3-P3m was then transferred into *A. hibisca* using the established methods [6]. The allelic replacement of the *orf1*, *orf30*, and *prmP3* genes in *A. hibisca* was confirmed following the method described previously [6].

RESULTS AND DISCUSSION

Identification and Sequence Analysis of the Pradimicin Gene Cluster

The pradimicin biosynthetic gene cluster of *A. hibisca* was identified from a fosmid library of genomic DNA. A fosmid clone harboring the pradimicin gene cluster (pBC3) was identified by using the ketosynthase genes obtained by PCR amplification as probes. The fosmid clone was used as a probe to identify the other overlapping fosmid clone pBC4. A region of 39 kb comprising two overlapping pBC3 and pBC4 fosmid clones was sequenced and analyzed for the presence of open reading frames (ORFs) by use of the SeqMan II software package and BLAST program to

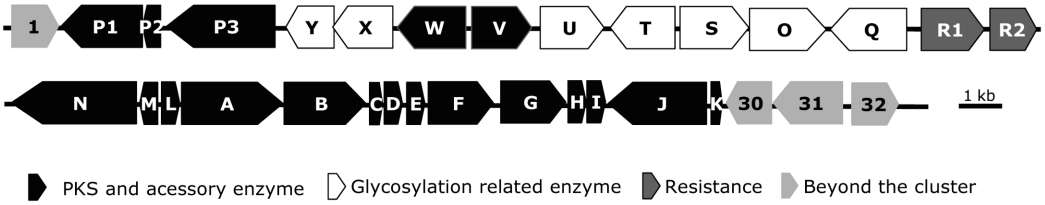

Fig. 2. Gene cluster map of the pradimicin biosynthetic genes of *A. hibisca* P157-2. Proposed functions of individual ORFs are summarized in Table 1.

compare with proteins in the databases. The sequence has been deposited at the NCBI database under the accession number EF151801. As the result of sequence analysis, 32 complete ORFs were identified (Fig. 2). The G+C content of this DNA region was 73.2% as the typical *Streptomyces* G+C bias content. The putative functions of each ORF and their closest homologs are shown in Table 1. Analysis of the gene products revealed strong similarities to the previously validated polyketide gene clusters of angucyclic antibiotics such as rubromycin, griseorhodin, and fredericamycin. The DNA sequences of several genes (*prmA~prmK*) of the *prm* gene cluster obtained in this report were identical to the corresponding gene sequences reported previously in *A. hibisca* [5]. PrmA, B, and C products showed strong similarities (89, 82, and 71% identity, respectively) with those of the ORF1, ORF2, and ORF3 identified from *A. verrucosospora* [4].

Genes Putatively Involved in the Biosynthesis of the Polyketide Moiety

Sequence analysis revealed the existence of the type II minimal polyketide synthase homologous genes *prmA*, *prmB*, and *prmC*, which encode KS_α, KS_β, and acyl carrier protein (ACP), respectively. The enzyme complexes would catalyze the biosynthesis of the polyketide backbone, which is later regiospecifically folded and cyclized by cyclase enzymes to form the characteristic angular structures. PrmA is highly similar to the KS_α (RubA) found from the rubromycin biosynthetic gene cluster [22]. All KS_αs contain active-site cysteine in a highly conserved sequence, STGCTSGLD. The same sequence was found in PrmA and contains Cys176 at the presumed active site. PrmB showed a high level of similarity to the KS_β of chartreusin PKS [36] and contains the characteristic Gln169 residue. KS_β is similar to KS_α but lacks the active site Cys, which is replaced by Gln. The *prmC* gene is located downstream of *prmB* and encodes an acyl carrier protein with the characteristic conserved G(x)DS motif, which is involved in the 4'-phosphopantetheine attachment for the post-translational modification of the enzyme [16]. These three enzymes comprise the minimal PKS and would be responsible for the assembly of eleven malonyl-CoA extender units and an initial acetyl-CoA starter unit through Claisen condensations for the generation of the dodecaketide (Fig. 1).

The pradimicin gene cluster contains three possible cyclase genes, *prmD*, *prmL*, and *prmK*, whose products are thought to be responsible for subsequent intramolecular aldol reactions, and for producing the stepwise ring closures that lead to the angular pentacyclic molecular structure (Fig. 1). PrmD shows high sequence similarity to the N-terminal region of RubF, GrhT, and TcmN. The RubF, GrhT, and TcmN are bifunctional cyclases that consist of two halves, being homologous to cyclases and oxidoreductases/methyltransferases. They are responsible for initial folding of the nascent linear polyketide intermediate and subsequent cyclizations involved in the formation of the aromatic rings [18, 22, 29]. The deduced protein PrmD is similar to those cyclases and possibly has a similar role in pradimicin biosynthesis, catalyzing the initial folding of the linear dodecaketide intermediate. PrmK shows a similarity to JadI (49% identity) of the jadomycin PKS. The angucycline cyclase JadI is responsible for catalyzing the cyclization of the fourth ring (the final ring of jadomycin) [15]. PrmL shares sequence similarity to FdmE (67% identity), GrhS (58% identity), and TcmJ (55% identity). The roles of FdmE, GrhS, and TcmJ are not clearly understood, but they seemingly enhance the yields of cyclized products through stabilization of the minimal PKS and cyclase protein complex [1, 18].

The product of *prmG* contains a short-chain alcohol dehydrogenase domain and shares high sequence similarity to RubG, a putative reductase gene. PrmG is probably involved in the reduction of the keto group at C11 of the nascent polyketide chain to a hydroxy group, a modification required for the subsequent cyclization [22].

PrmF shares high sequence similarity to the C-terminal region of TcmN (46% identity), which functions as an *O*-methyltransferase to catalyze the *O*-methylation of the phenolic OH group of tetracenomycin [29]. By analogy, PrmF presumably is the methyltransferase responsible for the *O*-methylating OH group of ring A of pradimicin (Fig. 1). The methyltransferase homologous to PrmF could also be found in the fredericamycin (FdmN, 50% identity), griseorhodin A (GrhL, 24% identity), and rubromycin (RubO, 29% identity) biosynthetic pathways, all of which

Table 1. Proposed function of ORFs in the pradimicin biosynthetic gene cluster.

Gene	Size (a.a.)	Protein homologs (the accession numbers)	Identity (%)	Proposed function
prmP1	449	ZhuD (AAG30191) *Streptomyces* sp. R1128	63	Biotin carboxylase
		AccC (YP713409) *Frankia alni*	64	
prmP2	154	AccB (YP713408) *Frankia alni*	70	Biotin carboxyl carrier protein
		ZhuE (AAG30192) *Streptomyces* sp. R1128	70	
prmP3	554	AccA (YP713407) *Frankia alni*	62	Transcarboxylase
		ZhuF (AAG30193) *Streptomyces* sp. R1128	58	
prmY	331	RubN2 (CAI94677) *S. achromogenes*	68	dNDP-D-glucose 4,6-dehydratase
		Gdh (AAK83290) *S. spinosa*	65	
prmX	356	SchS6 (CAF31364) *Streptomyces SCC 2136*	56	dNDP-1-glucose synthase
		Gra-ORF16 (CAA09637) *S. violaceoruber*	56	
prmW	402	CYP881 (CAE53712) *S. peucetius*	42	Oxygenase
		CYP (AAD28449) *S. lavendulae*	41	
prmV	332	Gra-ORF29 (CAA09650) *S. violaceoruber*	54	Oxygenase
		SAV6641 (NP827817). *S. avermitilis MA-4680*	48	
prmU	377	NmuI (YP410988) *Nitrosospira multiformis*	56	Aminotransferase
		EffA (YP606036) *Pseudomonas entomophila*	54	
prmT	340	TcmO (P39896) *S. glaucescens*	49	Methyltransferase
		GrhL (AAM33664) *Streptomyces JP95*	45	
prmS	438	RubG (CAC93713) *Lachevalieria aerocolonigenes*	38	Glycosyltransferase
		AtG (ABC02800) *A. melliaura*	38	
prmO	239	AknX2 (AAF73460) *S. galilaeus*	53	Aminomethylase
		NbmE (AAM88354) *S. narbonensis*	55	
prmQ	436	GT family 28 (YP823260) *Solibacter usitatus*	41	Glycosyltransferase
		GT family 28 (YP823259) *Solibacter usitatus*	39	
prmR1	348	CmrA (CAE17540) *S. griseus*	46	ABC transport system ATP-binding protein
		MtrA (CAK50797) *S. argillaceus*	44	
prmR2	260	SAV6712 (NP827888) *S. avermitilis*	47	ABC-type multidrug transport system
		TVN0936 (NP111455) *Thermoplasma volcanium*	28	
prmN	621	FdmV (AAQ08933) *S. griseus*	48	Asparagine Synthetase
		RubR (AAM97368) *S. collinus*	47	
prmM	154	Rxyl (YP645372) *Rubrobacter xylanophilus*	40	Unknown
		hypothetical protein (NP828636) *S. avermitilis*	38	
prmL	148	FdmE (AAQ08915) *S. griseus*	67	Cyclase
		GrhS (AAM33682) *Streptomyces JP95*	58	
prmA	426	RubA (AAG03067) *S. collinus*	75	KS_{α}
		AspA (AAV48829) *S. spiramyceticus*	75	
prmB	393	ChaB (CAH10162) *S. chartreusis*	62	KS_{β}
		OvmK (CAG14966) *S. antibioticus*	62	
prmC	88	ZhuN (AAG30201) *Streptomyces* sp. R1128	55	ACP
		RubC (AAG03069) *S. collinus*	41	
prmD	154	RubF (AAG03070) *S. collinus*	67	Cyclase
		GrhT (AAM33685) *Streptomyces JP95*	60	
prmE	154	FdmM1 (AAQ08924) *S. griseus*	43	Oxygenase
		GrhM (AAM33665) *Streptomyces JP95*	37	
prmF	342	TcmN (P16559) *S. glaucescens*	46	Methyltransferase
		FdmN (AAQ08925) *S. griseus*	50	
prmG	247	RubG (AAG03071) *S. collinus*	53	Reductase
		GrhO10 (AAM33668) *Streptomyces JP95*	52	
prmH	114	GrhU (AAM33683) *Streptomyces JP95*	63	Oxygenase
		RubH (AAG03072) *S. collinus*	65	
prmI	104	FdmQ (AAQ08928) *S. griseus*	46	Oxygenase
		RubT (AAM97374) *S. collinus*	41	
prmJ	412	CYP881 (CAE53712) *S. peucetius*	47	Oxygenase
		LnmA (AAN85514) *S. atroolivaceus*	47	
prmK	115	JaDI (AAD37852) *S. venezuelae*	49	Cyclase
		GrhQ (AAM33678) *Streptomyces JP95*	41	

produce a polyketide moiety having a methoxyquinone structure [18, 34].

At least three tailoring oxygenations appear to take place during pradimicin biosynthesis. The oxygens at C6, C8, and C20 do not originate from malonyl-CoA precursor but are probably generated by monooxygenases. Three monooxygenases (PrmJ, PrmW, and PrmV) were found in the pradimicin gene cluster. PrmJ shares a high level of similarity to LnmA, which is a cytochrome P450 hydroxylase identified in the leinamycin gene cluster of *S. atroolivaceus* [3]. PrmW is similar to the cytochrome P450 hydroxylases found from the genome sequence of *S. peucetius* ATCC27952 [23] and the mitomycin biosynthetic gene cluster of *S. lavenulae* [21]. PrmV also contains the domain of FMN-dependent monooxygenases, similar to the enzyme that is obtained from the granaticin biosynthetic gene cluster of *S. vioaceoruber* Tu22 [30].

PrmN shares a high level of sequence similarity to a family of asparagine synthetases, such as FdmV and RubR. FdmV has been proposed to be involved in transferring an amino group from an amino acid to the fredericamycin intermediate to afford the 2-pyridone moiety [34]. PrmN is possibly responsible as well for transferring an amino acid into the polyketide intermediate.

Three ORFs within the pradimicin gene cluster encode proteins whose function cannot be predicted by sequence comparison alone. PrmE, PrmH, and PrmI contain a so-called "antibiotic monooxygenases domain" identified from several *Streptomyces* sp. [34]. These genes (*prmE*, *prmH*, *prmI*) have close homologs in the fredericamycin, rubromycin, and griseorhodin gene cluster. Given the structural and biosynthetic resemblance among pradimicin, fredericamycin, rubromycin, and griseorhodin, these findings suggest that such genes may play roles in the common post-PKS oxidative tailoring steps [34].

Genes Putatively Involved in Sugar Biosynthesis

The genes *prmQ*, *prmO*, *prmS*, *prmT*, *prmU*, *prmX*, and *prmY* appear to encode the enzymes related to sugar biosynthesis and the subsequent glycosylation of pradimicin. PrmY is most similar to dNDP-glucose 4,6-dehydratase from the rhamnose biosynthesis pathway of *Saccharopolyspora spinosa* [20]. PrmX resembles Gra-ORF16 (56% identity), a dNDP-glucose synthase found from the granaticin biosynthetic gene cluster of *Streptomyces violaceoruber* [30]. These enzymes have been shown to catalyze the first two conserved steps in the formation of dNDP-4-keto-6-deoxy-glucose [20]. PrmU and PrmO resemble an aminotransferase and aminomethylase, respectively, which were found in a deoxyaminosugar biosynthetic gene cluster, and are probably involved in production of the thomosamine moiety. The presence of two glycosyltransferases in the cluster is consistent with the fact that the pradimicin aglycone is substituted by two different sugar moieties, thomosamine and xylose (Fig. 1). PrmS shows a high level of similarity to the glycosyltransferase (RubG) found in the rubromycin biosynthetic gene cluster of *Streptomyces* sp. The PrmQ most resembles a glycosyltransferase from *Solibacter usitatus*.

Other Genes

PrmR1 and PrmR2 are most similar to the amino acid sequences of a variety of known transporter genes. PrmR1 resembles the proteins of the daunorubicin-resistant ABC transporter ATP-binding protein family, whereas PrmR2 shows a high degree of similarity to several ABC-type multidrug transport systems. These genes are putatively involved in pradimicin resistance, most probably via metabolite export pumps powered by transmembrane electrochemical gradients [34].

We found the presence of PrmP1, PrmP2, and PrmP3, which are expected to be involved in the synthesis of a malonyl-CoA extender unit. The PrmP1 and PrmP3 are very similar to the α-chain (biotin carboxylase) and β-chain (transcarboxylase) of the acetyl-CoA carboxylase complex. PrmP2 resembles biotin carboxyl carrier proteins. PrmP1 is thought to act as a biotin carboxylase that incorporates CO_2 into a biotin carrier protein. PrmP3 would then catalyze the transcarboxylation from biotin-CO_2 to acetyl-CoA, thereby providing the malonyl-CoA precursor [8]. Other angucycline antibiotic gene clusters such as jadomyin [33], landomycin [35], and simocyclinone [31] also contain genes that are similar to *prm1*, *prm2*, and *prm3*.

Confirmation of the Pradimicin Gene Cluster by Gene Inactivation

In order to confirm that the cloned genes were essential for pradimicin biosynthesis, an in-frame deletion mutant of *A. hibisca* was constructed (Fig. 3). The pBC3-based plasmid pBC3m was created by replacing *prmA* and *prmB* encoding KS_α and Ks_β with the *aac(3)IV* apramycin-resistant gene and *oriT* cassette by the PCR-targeted *Streptomyces* gene replacement method [6]. The pBC3m was transferred to wild-type *A. hibisca* by conjugation, and double crossover mutants were selected (Fig. 3). HPLC analysis revealed that the mutant completely lost its ability to produce pradimicins, whereas wild-type strain produced 9.1 mg/l of pradimicin A and 5.4 mg/l of pradimicin B (Fig. 3). This result is a direct evidence for the involvement of the cloned genes in the biosynthesis of pradimicin.

The pradimicin gene cluster boundaries were defined by inactivation of selected genes (*orf1* and *orf30*) residing at the distal ends of the sequenced region. The gene *orf1* residing upstream of *prmP1* encodes the protein that resembles the 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-succinyltransferase (ZP00571119, 63% identity) of *Frankia* sp. The gene of pBC4 was replaced with the apramycin-resistant gene and *oriT* by the PCR-targeted *Streptomyces*

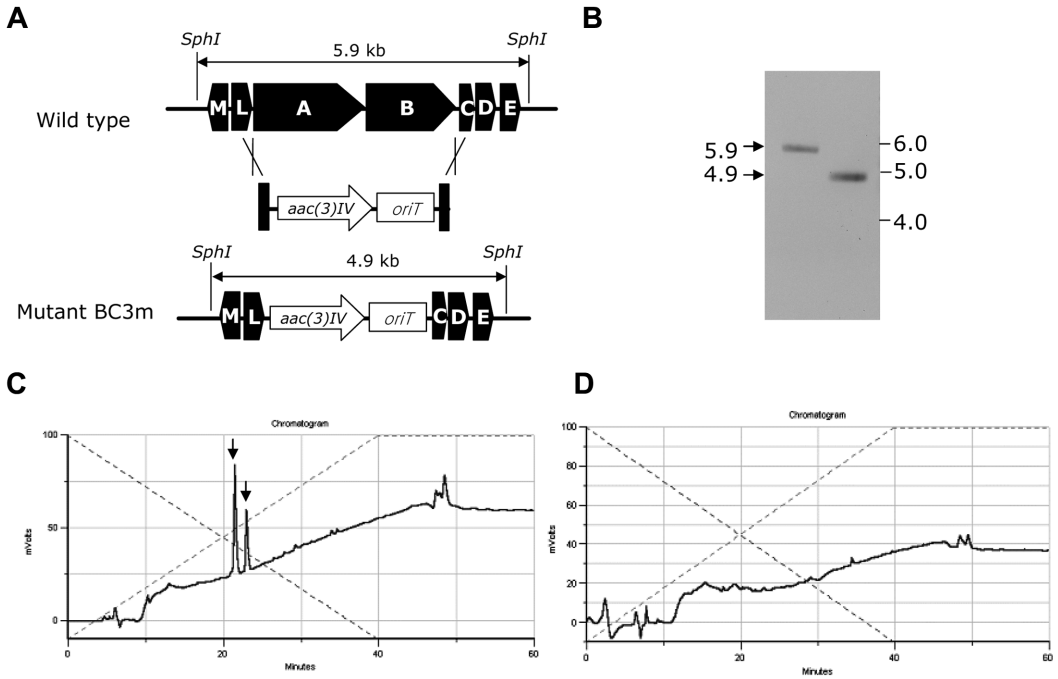

Fig. 3. Confirmation of the cloned cluster to encode pradimicin biosynthesis by inactivation of *prmA* and *prmB*.
A. Isolation of *prmAB* deletion mutant BC3m and restriction maps of the *A. hibisca* wild-type and the mutant strain showing predicted fragment sizes upon *SphI* digestion. **B**. Southern analysis of wild-type (lane 1) and BC3m (lane 2) genomic DNAs digested with SphI, using a 612-bp fragment of *prmL* as a probe. **C**. HPLC analysis of the culture extract of the wild-type strain. Arrows indicate pradimicin A and pradimicin B. **D**. HPLC analysis of the culture extract of the mutant strain BC3m.

gene replacement method. The resulting pBC4m was transferred into *A. hibisca* by conjugation to give a double crossover mutant. The mutant (BC4m) was selected in MS medium amended with apramycin. The mutant still produced pradimicin (8.9 mg/l pradimicin A and 5.8 mg/l pradimicin B) at the similar level as wild-type *A. hibisca*, which indicates that the deletion of the *orf1* gene has little effect on pradimicin production.

The gene *orf30* locating downstream of *prmK* was also replaced by the apramycin-resistant gene and *oriT* as described previously. Orf30 is highly similar to a hypothetical protein (NP733656, 42% identity) found from the genome sequence of *S. coelicolor*. The mutant strain (BC3m30) was fermented and examined for pradimicin production. The effect of mutation on the level of pradimicin was insignificant. The mutant produced 9.0 mg/l of pradimicin A and 5.1 mg/l of pradimicin B. These results indicate that *orf1* and *orf30* are either outside of the pradimicin gene cluster or dispensable under the growth conditions used in this study.

Effect of Gene Disruption of *prmP3*

The pradimicin polyketide moiety is generated by condensation of an acetyl-CoA starter unit with eleven C_2 extender units supplied from malonyl-CoA. Malonyl-CoA is required for chain elongation in all organisms that biosynthesize fatty acids not only for the polyketide synthesis. Malonyl-CoA is synthesized by carboxylation of acetyl-CoA. The carboxyl group is introduced through two distinct reactions catalyzed by a composite enzyme, acetyl-CoA carboxylase (EC 6.4.1.2), which consists of a biotin carboxylase (BC), a biotin carboxyl carrier protein (BCCP) with biotin as an essential cofactor, and a transcarboxylase (TC). The initial reaction, catalyzed by BC, uses bicarbonate to carboxylate BCCP; the second reaction, catalyzed by TC, transfers the carboxyl group from BCCP to acetyl-CoA, forming malonyl-CoA [28]. The organization of genes encoding the enzymes is variable in different organisms [28].

Most type II polyketide biosynthetic gene clusters do not contain the enzymes because the malonyl-CoAs are supplied by a primary metabolic pathway such as fatty acid biosynthesis [2]. An interesting feature of the pradimicin gene cluster is the presence of *prmP1*, *prmP2*, and *prmP3*, which are homologous to BC, BCCP, and TC. Other angucycline antibiotic gene clusters, such as jadomycin [33], landomycin [35], and simocyclinone [31], also contain genes encoding the acetyl-CoA carboxylase complex. The role of these gene products would be in supplying a malonyl-CoA precursor to the pradimicin biosynthetic pathway [33]. In order to know the effects of these genes, *prmP3* encoding transcarboxylase was deleted from the gene cluster as described previously (Fig. 4). The resulting mutant (BC3-P3m) was selected from MS medium and the production levels of pradimicins by the mutant were

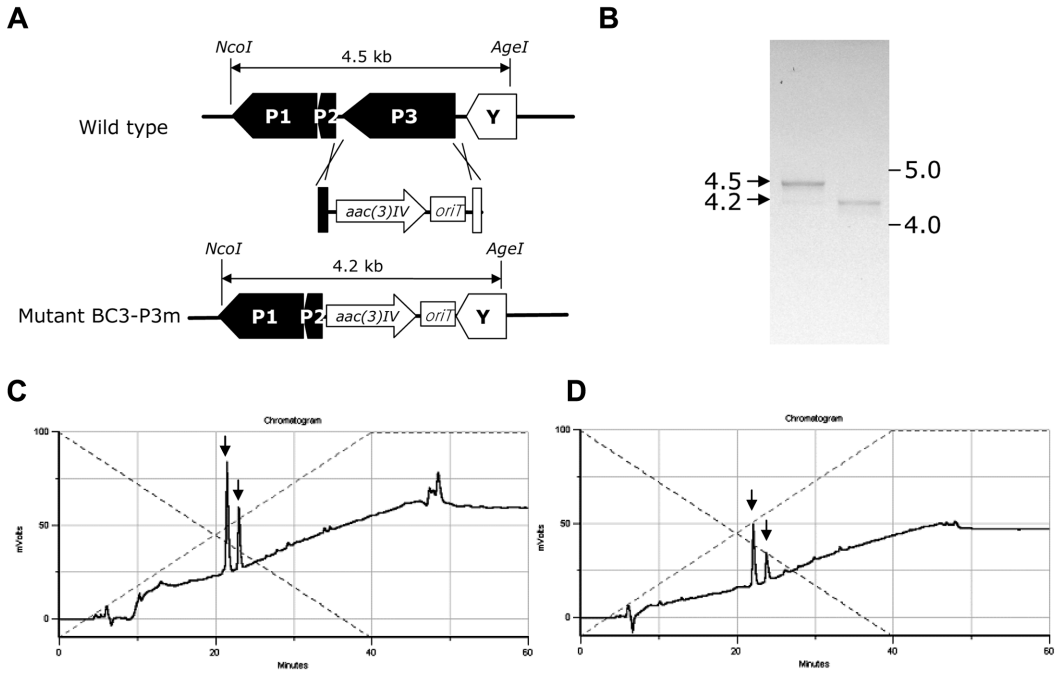

Fig. 4. Inactivation of the *prmP3* gene in the pradimicin biosynthetic gene cluster.
A. Isolation of *prmP3* deletion mutant BC3-P3m and restriction maps of *A. hibisca* wild-type and the mutant strain showing predicted fragment sizes upon NcoI and AgeI digestion. **B**. Southern analysis of wild-type (lane 1) and BC3-P3m (lane 2) genomic DNAs digested with NcoI and AgeI using an 825-bp fragment of *prmP1* as a probe. **C**. HPLC analysis of the culture extract of the wild-type strain. Arrows indicate pradimicin A and pradimicin B. **D**. HPLC analysis of the culture extract of the mutant strain BC3-P3m. Arrows indicate pradimicin A and pradimicin B.

compared with those by wild-type *A. hibisca*. The production levels of pradimicins by the mutant strain decreased to 62% of the level produced by the wild-type (Fig. 4). The mutant produced 5.6 mg/l of pradimicin A and 3.4 mg/l of pradimicin B. This result indicates that the *prmP3* product has a major role in the biosynthesis of the polyketide intermediate that is processed to form pradimicins. However, the pradimicin production was not completely inhibited in the *prmP3* deletion mutant, which may be due to metabolic leakage from the primary biosynthetic pathways. However, it is apparent that the *prmP3* homolog in the primary metabolic pathway is unable to fully complement the defect in the *prmP3* deletion mutant. In a previous report on jadomycin biosynthesis, the deletion of the acetyl-CoA carboxylase gene *jadJ* resulted in a 85% reduction of the jadomycin production [7]. These results indicate that the acetyl-CoA carboxylase gene *prmP3* would have a significant role in the production of pradimicin by supplying a precursor pool of malonyl-CoA.

We have confirmed that the cloned gene cluster was responsible for the production of pradimicin by using a mutant in which the gene encoding the PKS (KS_α and KS_β) was disrupted by in-frame deletion. Pradimicin is an unusual dodecaketide angucycline antibiotic compound. The type II polyketide synthases producing the angucycline structures larger than dodecaketide are the griseorhodin, fredericamycin, and rubromycin polyketide synthases. Although the angucycline structures of these compounds are further processed to generate a chiral spiro-carbon center [34], the fact that the pradimicin biosynthetic machinery shares similarities with the oxygenases and cyclases known for the griseorhodin, fredericamycin, or rubromycin biosynthetic machinery supports the suggestion of a common mechanism for forming the angucycline ring moiety. The availability of the pradimicin biosynthetic genes described in this report would provide new tools to study the unusual biochemistry of angucycline biosynthesis, and to refine the understanding of the cyclization mechanism, and also provide the tools for combinatorial biosynthetic studies to generate novel angucycline antibiotics.

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