

Cloning of the Pactamycin Biosynthetic Gene Cluster and Characterization of a Crucial Glycosyltransferase Prior to a Unique Cyclopentane Ring Formation

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Abstract The biosynthetic gene (*pct*) cluster for an antitumor antibiotic pactamycin was identified by use of a gene for putative radical *S*-adenosylmethionine methyltransferase as a probe. The *pct* gene cluster is localized to a 34 kb contiguous DNA from *Streptomyces pactum* NBRC 13433 and contains 24 open reading frames. Based on the bioinformatic analysis, a plausible biosynthetic pathway for pactamycin comprising of a unique cyclopentane ring, 3-aminoacetophenone, and 6-methylsalicylate was proposed. The *pctL* gene encoding a glycosyltransferase was speculated to be involved in an *N*-glycoside formation between 3-aminoacetophenone and UDP-*N*-acetyl- α -D-glucosamine prior to a unique cyclopentane ring formation. The *pctL* gene was then heterologously expressed in *Escherichia coli* and the enzymatic activity of the recombinant PctL protein was investigated. Consequently, the PctL protein was found to catalyze the expected reaction forming β -*N*-glycoside. The enzymatic activity of the PctL protein clearly confirmed that the present identified gene cluster is for the biosynthesis of pactamycin. Also, a glycosylation prior to cyclopentane ring formation was proposed to be a general strategy in the biosynthesis of the structurally related cyclopentane containing compounds.

Keywords biosynthesis, natural products, antibiotics, cyclitols, glycosyltransferase, pactamycin, radical SAM protein

Introduction

Pactamycin is an antitumor antibiotic produced by *Streptomyces pactum*, and in fact a potent inhibitor of translation in all three kingdoms eukarya, bacteria, archaea [1]. For the reason, pactamycin has mainly been used as a biochemical tool to study the protein synthesis. Recently, the complex structure of the 30S ribosomal subunit and pactamycin clearly revealed that it selectively interacts with the E site of the 30S subunit [2]. Apart from the bioactivity, pactamycin is structurally unique with a core cyclopentane ring which is decorated with 3-aminoacetophenone, 6-methylsalicylate, *N,N*-dimethylcarbamate, some alkyl, amino, and hydroxy groups (Fig. 1). Because of considerable interest for the biosynthesis of the complex molecule, feeding experiments into a culture of *S. pactum* were performed by Rinehart and coworkers, resulting in that the biosynthetic origins were derived from a complex biosynthetic pathway involving glucose, acetate, and methionine (Fig. 1) [3, 4].

The cyclopentane ring was thus found to be derived by a carbon–carbon bond formation between C-1 and C-5 of glucose [3]. This cyclization pattern of glucose/glucosamine has also been observed in the biosynthesis of the cyclopentane moieties [5] in allosamidine [6, 7] bacteriohopanetetrol [8], and calditol [9]. Therefore, similar enzymes seem to be involved in the construction of

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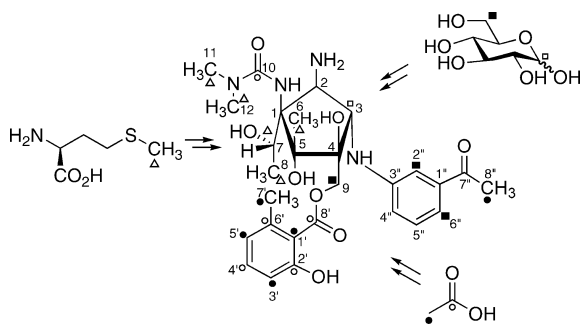


Fig. 1 Structure of pactamycin and summary of the previous feeding experiments.

The feeding experiments by use of [methyl- ^{13}C]methionine, [1- ^{13}C]acetate, [2- ^{13}C]acetate, [6- ^{13}C]glucose, and [1- ^{13}C]glucose were carried out by Rinehart and coworkers.⁴⁾

the cyclopentane rings in each biosynthetic pathway. However, such biosynthetic enzyme has never been identified. The 5-membered ring formation in the biosynthesis of pactamycin is thus a quite intriguing issue on the viewpoint of the carbocyclic formation from carbohydrate.

The biosynthesis of *m*-aminobenzoyl (C_7N) units has been extensively studied and the aminoshikimate pathway for the C_7N unit of rifamycin [10, 11], ansatrienin, naphthomycin [12], mitomycin C [13], has been established on the genetic and enzymatic level. Incorporation study of pactamycin revealed that 3-aminoacetophenone moiety is derived from glucose *via* probably 3-deoxy-D-arabinoheptulosonate 7-phosphate in the shikimate pathway [3, 4]. In contrast to the aminoshikimate pathway, the carbonyl group of dehydroshikimate (DHS) seems to be transaminated and dehydrated leading to 3-aminobenzoic acid (3-ABA), which condenses with acetate and further decarboxylates to afford 3-aminoacetophenone. Successful feeding experiments with 3-ABA and 5-fluoro-3-ABA giving corresponding pactamycin analogs clearly illustrated that the 3-aminoacetophenone moiety is constructed *via* 3-ABA [4, 14].

The 6-methylsalicylate (6-MSA) moiety of pactamycin is constructed through the polyketide pathway, since ^{13}C -labeled acetates were incorporated well into the unit. In actinomycetes, this type of simple aromatic polyketides is generally biosynthesized by an iterative type I polyketide synthase (PKS) [15], which has been quite recently established and are encoded in the avilamycin [16], calicheamicin [17], neocarzinostatin [18], chlorothricin [19], biosynthetic gene clusters. Therefore, an iterative type I PKS was also anticipated to be involved in the biosynthesis of pactamycin.

N,N-Dimethyl group of carbamate and the branching alkyl groups of the cyclopentane unit of pactamycin were labeled by feeding of the ^{13}C -labeled methionine. Of particular interest is the labeling of *C*-alkyl groups by methionine, a pattern observed previously in the hydroxyethyl moiety of carbapenem thienamycin [20], sitosterol, stigmasterol, and related 24-ethyl steroids [21].

By the above-mentioned feeding studies, the biosynthetic machinery for pactamycin seemed to contain a number of unprecedented biosynthetic enzymes for the construction of the complex molecule. This prompted us to study the biosynthesis of pactamycin on the genetic and enzymatic level. In the present paper, by using a characteristic probe of a gene for putative radical-*S*-adenosylmethionine (SAM) enzyme, we successfully identified the pactamycin biosynthetic (*pct*) gene cluster containing 24 open reading frames (*orfs*) expanded in 33.8 kbp. Further, a gene *pctL* encoding a putative glycosyltransferase was expressed in *Escherichia coli* and the function of the recombinant PctL protein was investigated.

Experimental

Strains

S. pactum NBRC13433 (pactamycin producer) was purchased from National Institute of Technology and Evaluation Biological Resource Center (Japan) as a source of genomic DNA including the pactamycin biosynthetic gene cluster and was cultured on Maltose-Bennett's Agar (0.1% Yeast extract, 0.1% beef extract, 0.2% NZ amine type A, 1.0% maltose and 2.0% agar, pH 7.3) or in its liquid medium. *E. coli* strain DH5 α was used as a host strain for routine subcloning of DNA fragments and was grown in Luria-Bertani (LB) medium supplemented with 50 $\mu\text{g}/\text{ml}$ of ampicillin or kanamycin as required. General DNA manipulations in *Streptomyces* and *E. coli* were performed according to the standard protocol [22, 23].

PCR Amplification of the Gene Encoding Fe-S Containing Radical SAM Methyltransferase

Genomic DNA obtained from cells of *S. pactum* NBRC13433 was used as a template for the PCR amplification of a part of the gene for Fe-S containing radical SAM methyltransferase. The newly designed degenerated primers methyl-F 5'-TGCCSSTTCWSSTG-CWSSTTCTG-3' and methyl-R 5'-GTCGAASKTGA-ASKTGTCGTC-3' were used for the PCR. K: a mixture of G and T. S: a mixture of C and G. W: a mixture of A and T. PCR conditions were 94°C, 5 minutes for denature, 30 cycles of 94°C, 30 seconds, 50°C, 30 seconds, 72°C, 20

seconds for extension of DNA in 5.0 μ l of 2 \times GC buffer I, 1.6 μ l of dNTP (2.5 mM each), 1.0 μ l of methyl-F (10 μ M), 1.0 μ l of methyl-R (10 μ M), 0.1 μ l of LA-Taq DNA polymerase (TaKaRa), and 1.3 μ l of template DNA (14 ng/ μ l of chromosomal DNA). The PCR products were sub-cloned with T vector of pT7-blue to obtain plasmids containing a partial sequence of the desired gene. The DNA sequences of the cloned plasmids were analyzed by use of a DNA sequencer. DNA sequence analysis was carried out with a LONG READER 4200 (Li-Cor) and a Thermo Sequenase Cycle Sequencing Kit according to the manufacturer's protocol.

Identification of the Pactamycin Biosynthetic Gene Cluster

The chromosomal DNA of *S. pactum* was partially digested with *Sau3AI* and the restriction enzyme was denatured with the phenol-chloroform extraction. After the ethanol precipitation, the digested DNA fragments of the chromosomal DNA were treated with Calf intestine alkaline phosphatase (CIAP) at 50°C and CIAP was denatured with the phenol-chloroform extraction. A cosmid vector pOJ446 was separately digested with *HpaI*, and after the treatment of CIAP, followed by digestion with *BamHI*. After the phenol-chloroform extraction and the ethanol precipitation, the resulting vector DNA was dissolved with the TE buffer. The digested pOJ446 and the partially digested chromosomal DNA were ligated by DNA Ligation kit ver2 (TaKaRa). After the ethanol precipitation, the ligated DNA was dissolved with the TE buffer. The resulting ligation mixture was packaged into λ phage followed by phage transfection to *E. coli* XL1 Blue MRF' by Gigapack III Gold Packaging Extract (Stratagene) according to the manufacturer's protocol. The host strain *E. coli* XL1 Blue MRF' was cultured in 3.0 ml of LB containing 10 mM MgSO₄ and 0.2% maltose by OD₆₀₀ 1~2. One ml of the culture was centrifuged and the resulting wet cells were suspended in a 10 mM MgSO₄ up to OD₆₀₀ 1 for transfection.

A cosmid library of 800 clones in *E. coli* was screened by the hybridization with a Dig labeled DNA probe, which was made by the DIG DNA Labeling Kit (Roche) from PCR amplified DNA fragments of the partial gene for radical SAM methyltransferase. The hybridization was carried out by the DIG Nucleic Acid Detection Kit with NBT/BCIP solution (Roche) according to the manufacturer's protocol.

A positive clone was cultured and the cosmid was extracted by a standard protocol. The cosmid was confirmed whether it contains a radical SAM methyltransferase gene by the PCR mentioned above and Southern hybridization

with the same probe. The desired cosmid (cpac8) was randomly sequenced by a shotgun sequence method on double-stranded DNA templates with more than 10-fold coverage and minimum 3 times each portion of the DNA sequence (Shimadzu Biotech). A cosmid cpac8-11 containing adjacent region of cpac8 was similarly obtained. The insert DNA of cpac8-11 was digested with some restriction enzymes and cloned into the plasmid vector LITMUS28. The obtained plasmids containing each portion of the DNA were sequenced on double-stranded DNA templates by using a LONG READER 4200 (Li-Cor). As a result, a 43 kbp of DNA sequence containing the *pct* cluster was determined. ORFs were determined by FramePlot analysis [24] (www.nih.go.jp/~jun/cgi-bin/frameplot.pl) and BLAST homology search through web considering a possible Shine-Dargano sequence. The DNA sequence data of the *pct* gene cluster in *S. pactum* NBRC13433 has been deposited to the DDBJ, EMBL, and GenBank databases under accession number AB303063.

Expression and Functional Analysis of the *pctL* Gene Encoding a Glycosyltransferase

The *pctL* gene was amplified by PCR with a plasmid containing the gene derived from cpac8 with primers pctL-F: 5'-GACGcAtATGGAAGCGACGCCCGCCCG-3' and pctL-R: 5'-CCACCACaAgCtTGCCGGCCCGGGGT-3'. PCR conditions were 30 cycles of 98°C, 10 seconds, 55°C, 5 seconds, 72°C, 80 seconds for extension of DNA in 2.0 μ l of 5 \times PrimeSTAR buffer, 0.8 μ l of dNTP (2.5 mM each), 0.5 μ l of DMSO, 0.3 μ l of pctL-F (10 μ M), 0.3 μ l of pctL-R (10 μ M), 0.1 μ l of PrimeSTAR polymerase (TaKaRa), 1.0 μ l of template DNA, and 5.0 μ l of water. The amplified PCR product was digested with *HindIII* and the resulting DNA fragment was subcloned into the LITMUS28 (New England Biolabs) previously digested with *EcoRV* and *HindIII* to obtain LITMUS28-pctL. After confirmation of the DNA sequence, the appropriate plasmid was digested with *NdeI* and *HindIII* and the resulting DNA fragment was inserted into the corresponding site of pET30b(+) (Novagen) to obtain pET-pctL. The pET-pctL was introduced into *E. coli* BL21(DE3) by a standard chemical transformation. The *E. coli* harboring pET-pctL was grown in LB medium supplemented with 50 μ g/ml of kanamycin at 37°C by OD₆₀₀ 0.7~1.0 and a final 0.1 mM isopropyl β -D-thiogalactoside was added for induction of overexpression. The culture was continued at 37°C for several hours and the cells were harvested by centrifugation (6,000 rpm \times 30 minutes). The wet cells were suspended in a 50 mM Tris buffer (pH 8.0) containing 10% glycerol and 1.0 mM of MgCl₂, and disrupted by sonic oscillation. After centrifugation (10,000 rpm \times 30 minutes), the supernatant

was used as a cell free extract including the expressed PctL protein. A cell free extract of *E. coli* without the *pctL* gene (only pET30 plasmid) was also prepared as a control. The PctL reaction (final 100 μ l) was carried out in 2.0 mM of 3-aminoacetophenone, 2.0 mM of UDP-GlcNAc, and the CFEs (PctL or empty pET30) at 28°C for 4 hours. By addition of methanol (100 μ l), the enzyme reaction was quenched and the protein precipitate was removed by centrifugation. The resulting supernatant was injected onto a HPLC system with a Hitachi L-6250 Intelligent Pump, a L-4000H UV Detector and a D-2500 Chromato-Integrator. Senshu Pak, PEGASIL ODS (4.6 \times 250 mm) was used as a separation column and 25% aq methanol was used as elution solvent (0.9 ml/minute). Absorbance at 338 nm was monitored by HPLC. The PctL reaction product (200 μ l scale, 24 hours of the reaction time) was purified by HPLC and compared with the authentic compound by NMR (DRX-500, Bruker) and FAB-MS (JMS-700, JEOL).

Preparation of the Authentic β -N-Glycoside

N-Acetyl-D-glucosamine (2.0 mmol, 442 mg) and 3-aminoacetophenone (2.0 mmol, 135 mg) were mixed in 15 ml of methanol and 5.0 ml of acetic acid and the mixture was stirred at room temperature overnight. Acetic acid was necessary for the glycosylation as an acid catalyst. After removal of the solvent, the residue was directly purified by a standard silica-gel chromatography using CHCl₃/CH₃OH (20:1~6:1). The glycosylated product was recrystallized from the methanol solution to give 290 mg (43%) of pure compound as colorless crystal. Data: mp 192~194°C; $[\alpha]_D^{28}$ -60.1 (*c* 0.360, CH₃OH); IR (KBr): 3418, 3372, 3294, 2942, 2899, 1667, 1640, 1608, 1585, 1546, 1526, 1438, 1370, 1308, 1244, 1068, 1047, 786 cm⁻¹; UV $\lambda_{\max}^{\text{CH}_3\text{OH}}$ (ϵ): 233 (34,500), 336 (2,450); ¹H-NMR (500 MHz, CD₃OD): δ 1.98 (s, 3H), 2.54 (s, 3H), 3.40 (m, 2H), 3.54 (dd, *J*=8.5, 10 Hz, 1H), 3.68 (dd, *J*=5.1, 12 Hz, 1H), 3.84 (t, *J*=10 Hz, 1H), 3.85 (t, *J*=12 Hz, 1H), 4.62 (d, *J*=10 Hz, 1H), 6.92 (ddd, *J*=0.9, 2.2, 8.0 Hz, 1H), 7.24 (t, *J*=7.9 Hz, 1H), 7.27 (t, *J*=2.2 Hz, 1H), 7.33 (d, *J*=0.9, 8.0 Hz, 1H); ¹³C-NMR (125 MHz, CD₃OD): δ 23.58, 27.58, 57.63, 63.51, 73.05, 77.26, 79.26, 87.25, 114.82, 120.52, 120.59,

131.17, 140.01, 148.94, 175.83, 201.88; HR-MS (FAB, glycerol) *m/z* 339.1564 (M+H)⁺, calcd for C₁₆H₂₃N₂O₆: 339.1556; Element Analysis: Calcd. for C₁₆H₂₂N₂O₆: C 56.80, H 6.55, N 8.28, Found: C 56.55, H 6.72, N 8.35.

Results and Discussion

Cloning of the Pactamycin Biosynthetic Gene (*pct*) Cluster from *S. pactum* NBRC 13433

It was hypothesized that the biosynthesis of pactamycin would involve a unique *C*-methyltransferase. This hypothesis was based on several biosynthetic gene clusters for secondary metabolites including fosfomycin [25~28], bialaphos [29, 30], thienamycin [31], gentamicin [32], fortimicin [33, 34], because putative radical-SAM proteins encoded in these gene clusters were proposed to be involved in the *C*-methylation and *P*-methylation reactions [35]. A family of radical-SAM protein is an emerging group of enzyme, which triggers several radical reactions via a 5'-deoxyadenosine radical derived from *S*-adenosyl L-methionine [36~40]. So far, only a few of enzymes in primary metabolism have been characterized, because SAM has been known primarily just a biological methylating agent. Identification and characterization of such a unique radical-SAM methyltransferase in secondary metabolism are expected to contribute to elucidation of the enzymatic radical reactions. Also, this type of enzyme may be useful tool to modify biologically active molecules for the improvement of the activities.

A pair of PCR primers was designed to amplify a part of gene for the radical-SAM methyltransferase. Highly conserved sequences CPFSCSFC for the *N*-terminal region and DDN(T)FT(N)FD for the *C*-terminal region among BcpD, Fms7, Fom3, GacD, and SAV849, which are probable radical-SAM methyltransferases, were used to design the primers methyl-F 5'-TGCCCSSTTCWSSTG-CWSSTTCTG-3' and methyl-R 5'-GTGCGAASKTGAA-SKTGTGCTG-3', respectively. The PCR with the primers using a chromosomal DNA derived from *S. pactum* NBRC 13433 as a template successfully gave a fragment of gene

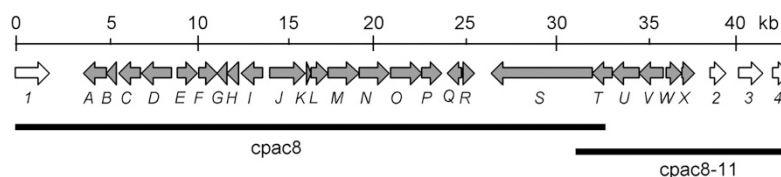


Fig. 2 Genetic organization of the *pct* gene cluster.

The *pct* gene cluster is shown in grey. Proposed functions of individual *orfs* are summarized in Table 1. Obtained cosmids cpac8 and cpac8~11 are also shown.

(ca. 150 bp) for a putative radical-SAM methyltransferase. Screening of a cosmid library of *S. pactum* using a DIG labeled probe for the gene lead to identification of a cosmid containing the gene for the radical-SAM methyltransferase. The whole region of the cosmid (cpac8) was then

sequenced, and further a cosmid (cpac8-11) containing an adjacent region was also cloned and sequenced (Fig. 2). Consequently, a gene cluster consisting of 28 *orfs* containing three genes encoding putative radical-SAM methyltransferase has been determined. The deduced

Table 1 Deduced functions of *orfs* in the pactamycin gene (*pct*) cluster

Gene	Size (aa)	Sequence similarity (homolog, origin)	Identity/ similarity (%)	Proposed function
<i>orf1^a</i>	>626	ATP-dependent RNA helicase (NP_825300), <i>S. avermitilis</i> MA-4680	70/77	ATP-dependent RNA helicase
<i>pctA</i>	397	cytochrome P450 monooxygenase (AAT45281), <i>S. tubecidicus</i>	59/71	cytochrome P450 hydroxylase
<i>pctB</i>	162	glutathione peroxidase (ZP_00616302), <i>Kinococcus radiotolerans</i>	60/72	glutathione
<i>pctC</i>	391	aminotransferase (ZP_01532175), <i>Roseiflexus castenholzii</i>	43/54	aminotransferase
<i>pctD</i>	570	carbamoyltransferase (MmcS, AAD32734), <i>S. lavendulae</i>	47/59	carbamoyltransferase
<i>pctE</i>	367	predicted Fe-S Radical SAM oxidoreductase (MitD, AAD32720), <i>S. lavendulae</i>	32/50	Fe-S Radical SAM oxidoreductase
<i>pctF</i>	358	putative methyltransferase (HemK family, CAJ88518), <i>S. ambofafiens</i>	35/49	N-methyltransferase
<i>pctG</i>	178	hypothetical protein (YP_001107758), <i>Saccharopolyspora erythraea</i>	38/59	unknown
<i>pctH</i>	216	two component transcriptional regulator, winged helix family (YP_611468) <i>Silicibacter</i> sp. TM1040	38/52	unknown
<i>pctI</i>	378	putative deacetylase (MitC, AAD32721), <i>S. lavendulae</i>	38/46	deacetylase
<i>pctJ</i>	661	putative Cobalamin B12-binding/Radical SAM methyltransferase (CAD18979), <i>S. cattleya</i>	32/47	C-methyltransferase
<i>pctK</i>	94	putative acyl carrier protein (YP_473450), <i>Clostridium perfringens</i>	33/61	discrete ACP
<i>pctL</i>	318	glycosyltransferase (MitB, AAD32722), <i>S. lavendulae</i>	34/43	glycosyltransferase
<i>pctM</i>	562	ketoacyl-ACP synthase (YP_001102877), <i>Saccharopolyspora erythraea</i>	44/55	ketoacyl-ACP synthase
<i>pctN</i>	575	Radical SAM binding protein (YP_485691), <i>Rhodopseudomonas palustris</i> HaA2	32/47	C-methyltransferase
<i>pctO</i>	578	Radical SAM binding protein (YP_485691), <i>Rhodopseudomonas palustris</i> HaA2	31/46	C-methyltransferase
<i>pctP</i>	351	putative NAD dependent dehydrogenase (YP_310737), <i>Shigella sonnei</i> Ss046	33/50	NAD dependent dehydrogenase
<i>pctQ</i>	257	possible hydrolase (YP_706151), <i>Rhodococcus</i> sp. RHA1	40/52	hydrolase
<i>pctR</i>	211	enterobactin synthetase component D (NP_541058), <i>Brucella melitensis</i> 16M	36/54	phosphopantetheinyl transferase
<i>pctS</i>	1861	iterative type I PKS (ChlB1, AAZ77673), <i>S. antibioticus</i>	53/63	iterative type I PKS: KS-AT-DH-KR-ACP
<i>pctT</i>	358	ketoacyl-ACP synthase (CalO4, AAM70354), <i>Micromonospora echinospora</i>	41/59	ketoacyl-ACP synthase
<i>pctU</i>	505	putative AMP-forming acyl-CoA synthetase (ZP_01196073) <i>Xanthobacter autotrophicus</i> Py2	32/43	acyl-CoA synthetase
<i>pctV</i>	444	glutamate-1-semialdehyde 2,1-aminomutase (ZP_01092129), <i>Blastopirellula marina</i> DSM 9941	40/55	aminotransferase
<i>pctW</i>	279	short-chain dehydrogenase/reductase (YP_643317), <i>Rubrobacter xylanophilus</i>	40/55	dehydrogenase/reductase
<i>pctX</i>	226	phosphoglycerate mutase (ZP_01515050), <i>Chloroflexus aggregans</i> DSM 9485	37/53	phosphatase
<i>orf2^a</i>	271	hypothetical protein (NP_824863) <i>S. avermitilis</i> MA-4680	46/55	hypothetical protein
<i>orf3^a</i>	431	integral membrane protein (NP_628287), <i>S. coelicolor</i> A3(2)	58/68	integral membrane protein
<i>orf4^a</i>	>311	probable peptidase (NP_628288), <i>S. coelicolor</i> A3(2)	52/60	probable peptidase

^a *orfs* probably beyond the *pct* cluster.

Table 2 Comparison of pactamycin and mitomycin C biosynthetic genes

<i>pct</i> genes	<i>mit</i> genes	Identity/similarity (%)	Possible function
<i>pctA</i>	<i>orf3, orf4</i>	40/55, 39/53	cytochrome P450
<i>pctC</i>	<i>mitA</i>	36/46	PLP dependent protein
<i>pctD</i>	<i>mmcS</i>	47/60	carbamoyltransferase
<i>pctE</i>	<i>mitD</i>	32/50	Fe-S oxidoreductase
<i>pctF</i>	<i>mitM</i>	partly	methyltransferase
<i>pctI</i>	<i>mitC</i>	38/46	deacetylase
<i>pctJ</i>	<i>mmcD</i>	28/41	Radical SAM methyltransferase
<i>pctK</i>	<i>mmcB</i>	31/62	ACP
<i>pctL</i>	<i>mitB</i>	34/43	glycosyltransferase
<i>pctN</i>	<i>mmcD</i>	29/42	Radical SAM methyltransferase
<i>pctO</i>	<i>mmcD</i>	29/44	Radical SAM methyltransferase
<i>pctP</i>	<i>mitG</i>	31/44	oxidoreductase
<i>pctU</i>	<i>mitE</i>	30/38	AMP dependent acyl CoA ligase
<i>pctW</i>	<i>mitF</i>	31/42	Ketoacyl reductase

functions of the *orfs* in the pactamycin gene cluster (*pct*) by homology analysis are shown in Table 1. The DNA sequence data for the *pct* cluster in this paper has been deposited to the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB303063.

Sequence analysis revealed that *orf1* encodes a putative ATP-dependant RNA helicase (>626 aa), and a 1926 bp non-coding region between *orf1* and *pctA* exists, indicating that *orf1* and its flanking non-coding region may be outside of the *pct* cluster. Due to the same reason, *orf2* appeared to be outside of the *pct* cluster. The homologous genes for *orf3* and *orf4* contiguously exist in *S. coelicolor* A3(2) as SCO4107 and SCO4108, respectively. These deduced gene products lack significant similarity to proteins involved in secondary metabolite biosynthesis. Therefore, these results, together with the functional assignment of deduced gene products within the sequenced region, support that the *pct* gene cluster would span 33.8 kb and encompass 24 *orfs* from *pctA* to *pctX*.

A pactamycin resistance determinant is known to be on a 4.9 kb *KpnI* fragment in the producer *S. pactum* [41, 42]. *S. lividans* harboring a plasmid containing the 4.9 kb *KpnI* fragment possess pactamycin resistant ribosome [41], because this fragment causes site-specific methylation of 16S rRNA [42]. Such 4.9 kb *KpnI* fragment with unique restriction enzyme sites is not involved in the present identified *pct* gene cluster. Thus, this pactamycin resistance determinant seems to be operated independently.

Some homologous genes were found in the biosynthetic gene cluster of antitumor mitomycin C derived from *S. lividans* (Table 2) [13]. Therefore, these gene products appeared to catalyze similar reactions in each

biosynthetic pathway and might be related to each other to construct complex molecules comprising of a C₇N, glucose/glucosamine, and carbamate unit.

The *orfs* Involved in the Biosynthesis of 3-Aminoacetophenone and Following Cyclopentane Ring Formation

Previous incorporation studies clearly indicated that 3-aminoacetophenone moiety is derived from the intermediates of the shikimate pathway. Among those, DHS was supposed to be a possible precursor from the incorporation pattern of ¹³C-labeled glucose. A presumable transamination of a carbonyl group in DHS would occur to form aminoshikimate, which could be aromatized by two dehydrations to give 3-ABA (Fig. 3A). Two genes, *pctC* and *pctV* encoding putative aminotransferases exist in the *pct* cluster and either of these seems to be involved in the presumed transamination. PctC shows homology to DegT/DnrJ/EryC1/StcC family of aminotransferase, which are known to be involved in the biosynthesis of the unique aminocyclitols streptomycin and 2-deoxystreptomycin (DOS) of the aminoglycoside antibiotics, streptomycin, butirosin, kanamycin, and neomycin [43, 44]. 3-Amino-5-hydroxybenzoic acid (AHBA) synthase, which is a pyridoxal 5'-phosphate (PLP) dependent enzyme catalyzing the α,β -dehydration and stereospecific 1,4-enolization of aminoDHS to AHBA in the aminoshikimate pathway, shows homology to this family of aminotransferase [45~47]. Therefore, PctC is most likely to be involved in the transamination of DHS and also in the two dehydrations as shown in Fig. 3A. No dehydratase-like protein encoded in the *pct* cluster might support the proposed multifunctional role of the protein. Another

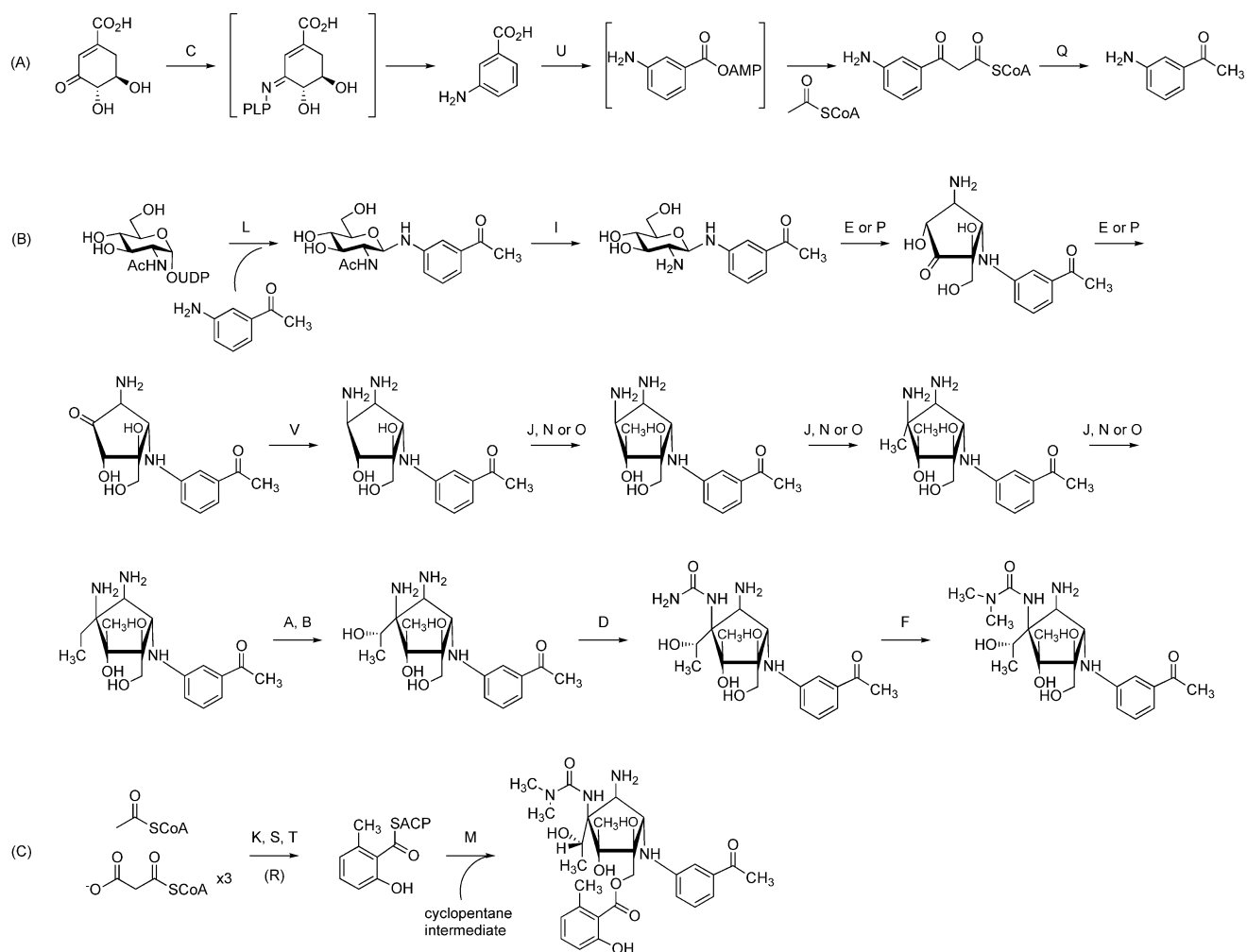


Fig. 3 Proposed biosynthetic pathway for pactamycin.

(A) Pathway for 3-aminoacetophenone, (B) a model assembly line of the cyclopentane ring, and (C) pathway for 6-MSA and completion of the biosynthesis. The order of the modification steps of cyclopentane ring can be interchangeable.

deduced aminotransferase PctV shows homology to glutamate-1-semialdehyde 2,1-aminomutases, which are also known as PLP-dependent aminotransferase. This type of protein in the secondary metabolism was found in the biosynthesis of DOS-containing antibiotic such as neomycin and butirosin and was reported to catalyze the transamination of pseudo-disaccharide 6'-oxo-paromamine in the biosynthesis of neamine [48]. Substrate structural relationship of homologous proteins support that the PctV protein might catalyze the other transamination rather than the transamination of relatively small DHS molecule.

The generated 3-ABA could be condensed with an acetyl CoA forming a probable β -ketoacyl CoA intermediate, which would be hydrolyzed and followed by decarboxylation to afford 3-aminoacetophenone. A putative

AMP forming acyl CoA synthetase, PctU, might catalyze the proposed initial reaction. PctU shows homology to the initial adenylation (A) domain of the RifA PKS in the biosynthesis of rifamycin. The A domain of RifA was reported to catalyze the adenylation of AHBA and loading it on the downstream peptidyl carrier protein in the PKS [49]. Structural similarity between 3-ABA and AHBA support the proposed function of PctU. The formed β -ketoacyl CoA would be hydrolyzed by a putative hydrolase PctQ and the β -keto-acid product would be decarboxylated leading to 3-aminoacetophenone. The generated 3-aminoacetophenone must be somehow connected with the core cyclopentane ring through a C–N linkage. An *N*-glycoside formation between 3-aminoacetophenone and glucose/glucosamine that is a direct precursor of the cyclopentane ring was presumed to be a possible

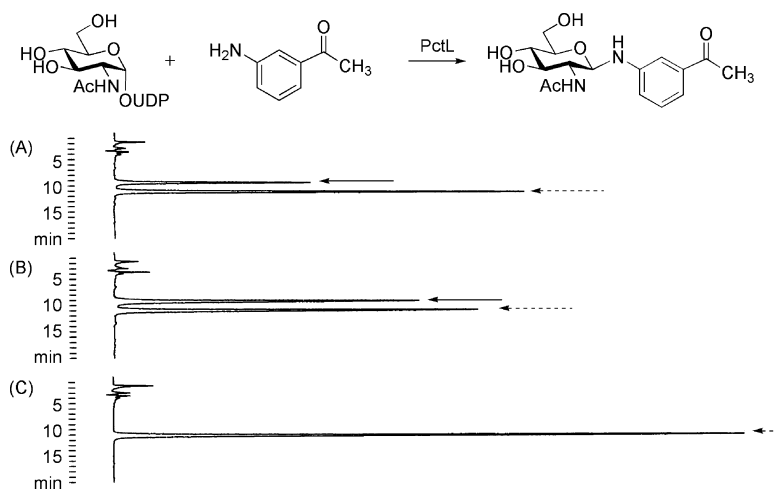


Fig. 4 Functional analysis of the PctL protein.

(A) HPLC chromatogram of products formed by the recombinant PctL protein in 50 mM Tris buffer (pH 8.0) containing 10% glycerol, 3-aminoacetophenone (2.0 mM), UDP-GlcNAc (2.0 mM) and $MgCl_2$ (1.0 mM), after incubation at 28°C for 4 hours. (B) HPLC chromatogram of products formed by an identical enzymatic incubation, co-injected with an authentic sample. (C) HPLC chromatogram of products formed by an identical enzymatic incubation with CFE of *E. coli* harboring empty expression plasmid pET30. The solid arrows indicate β -*N*-glycoside and the broken arrows indicate unreacted 3-aminoacetophenone.

biosynthetic assembly line (Fig. 3B). This enzymatic process was proposed in the bacteriohopanid biosynthesis [50]. In the case of the biosynthesis of pactamycin, a presumable *N*-glycoside intermediate would be constructed and opened up to form an iminium intermediate, which would lead to an intramolecular aldol reaction. A nucleophilic enolate can be generated by an oxidative *endo*-opening process at the C4 through equilibrium of keto-enol. A putative dehydrogenase encoded in the *pct* cluster, PctE or PctP, is thus presumed to be responsible for the proposed oxidation and cyclization. An *N*-glycoside formation prior to the cyclization seemed to be catalyzed by a putative glycosyltransferase PctL. To investigate this hypothesis, the *pctL* gene was overexpressed in *E. coli* and the enzyme reaction of the recombinant protein was conducted.

Functional Analysis of the *pctL* Gene Encoding a Glycosyltransferase

The *pctL* gene was expressed in *E. coli* by a standard manner and the recombinant PctL protein was obtained as a soluble form. UDP-*N*-acetyl- α -D-glucosamine (UDP-GlcNAc) was used as a possible glycosyl donor, because UDP-GlcNAc widely exists in bacterial cell and is used as a starter unit in the biosynthesis of bacterial peptidoglycan [51]. Also, the amino group at C2 in UDP-GlcNAc is corresponding to the amino group at C2 of pactamycin. *N*-Acetyl group of the glucosamine moiety derived from UDP-GlcNAc would be removed at certain step of the

biosynthesis by the deduced product of *pctL*, which shows homology to some possible deacetylases [52].

A cell free extract of *E. coli* containing the recombinant PctL protein was then mixed with 3-aminoacetophenone and UDP-GlcNAc. The enzyme reaction was quenched by addition of methanol and the supernatant was analyzed by HPLC (Fig. 4). A new peak was clearly observed in the PctL reaction, but not in the control reaction with a cell free extract of *E. coli* harboring empty expression plasmid pET30. The expected β -*N*-glycoside as the enzyme reaction product was separately prepared by treatment of *N*-acetyl-D-glucosamine and 3-aminoacetophenone under acidic conditions. As shown in Fig. 4, the PctL reaction product was co-eluted with the authentic compound. The enzymatic reaction product was further isolated by HPLC and the structure was confirmed by NMR and FAB-MS. Accordingly, the PctL protein was characterized to catalyze the *N*-glycoside formation with 3-aminoacetophenone using UDP-GlcNAc. This enzymatic activity of the PctL protein indicates that the present identified gene cluster is for the biosynthesis of pactamycin.

PctL belongs to family 2 glycosyltransferase (GT2), which is one of the largest families [53]. This family of glycosyltransferase is expected to use NDP- α -D-sugars to generate β -linked product. The observed enzymatic activity of the PctL protein coincides well with the reaction mechanism of GT2. MitB encoded in the mitomycin C biosynthetic gene cluster, which shows homology to PctL, could be also proposed to catalyze a glycosylation reaction

between the C₇N unit (AHBA) of mitomycin C and UDP-GlcNAc. As mentioned above, the *pct* genes and the mitomycin C biosynthetic genes share a number of homological genes. Therefore, the biosynthetic pathways for pactamycin and mitomycin C appear to be closely related each other. Functional characterization for either of the genes would give useful information to understand a true biosynthetic pathway.

The *orfs* Involved in the Biosynthesis of 6-MSA

Sequence analysis suggested that the products of five genes, *pctK*, *pctM*, *pctR*, *pctS*, and *pctT* were involved in the biosynthesis of the 6-methylsalicylic acid (6-MSA) moiety. The deduced product of *pctS* shows significant homology to many type I PKSs. PctS exhibits head-to-tail homology to ChlB1 encoded in the chlorothricin biosynthetic gene cluster [19] and these have the characteristic type I PKS domains, including a ketosynthase (KS), acyltransferase (AT), dehydratase (DH), ketoreductase (KR), and acyl carrier protein (ACP). Both PctS and ChlB1 show higher homology to some iterative type I PKSs including 6-methylsalicylic acid synthase (6-MSAS) derived from fungi. Since ChlB1 was confirmed to be involved in the 6-MSA formation [15, 19], PctS could also catalyze the formation of 6-MSA as an iterative type I PKS (Fig. 3C). The deduced product of *pctR* shows homology to 4'-phosphopantetheinyl transferases (pptases). PctR seems most likely to furnish the 4'-phosphopantethein group onto the apo form of ACPs to make mature proteins. A dedicated pptase in polyketide biosynthesis has recently identified from the fredericamycin producer *S. griseus* [54]. PctR would also act as a pathway specific pptase to modify the ACP in PctS and a putative discrete ACP, PctK.

PctT and PctM show homology to β -ketoacyl-ACP synthase III (KAS III, also FabH) and KAS I and II (mainly to FabB type protein) in the type II system of fatty acid biosynthesis (Fab), respectively. PctT is also highly homologous to ChlB3, ChlB6 [19], AviN [16], and CalO4 [17], all of which accompany with an iterative type I PKS. These homologous proteins were presumed to attach the starter unit, acetyl CoA, onto the corresponding PKSs, since FabH enzymes use an acyl-CoA rather than an acyl-ACP as the primer and catalyze the first condensation step in the pathway [55, 56]. PctT is thus proposed to catalyze the priming reaction with acetyl CoA onto a discrete ACP PctK, which would be specifically recognized by an iterative type I PKS PctS for the formation of 6-MSA. FabB enzymes serve a relatively large active site cavity and catalyze the condensation of a wide range of acyl-ACPs [57]. Therefore, its homologous protein PctM might recognize the attached 6-MSA-ACP thioester in the active

site of PctS, and catalyze a transfer reaction onto a hydroxy group in the core cyclopentane ring to form an ester bond (Fig. 3C). Alternatively, PctS might be self-priming an acetyl CoA for the 6-MSA synthesis, and the generated 6-MSA would be activated by PctT with a discrete ACP PctK to afford 6-MSA-ACP (PctK), which could be used for the transfer reaction by PctM. In any case, mechanistic characterization of the proteins, especially the modification enzyme of the cyclopentane ring, is important to manipulate the pactamycin biosynthesis for generation of the novel analogs.

The *orfs* Involved in the Modification of the Cyclopentane Ring

Although a precise order of the modification of the cyclopentane ring is unclear from current information of the *pct* gene cluster, a plausible model assembly line can be proposed as shown in Fig. 3B. The presumed cyclopentane intermediate might be somehow isomerized to its isomer through a presumable enediol intermediate by a putative dehydrogenase PctE or PctP. The generated ketone would be transaminated by a putative aminotransferase PctV, which may prefer a large molecule as a substrate as mentioned above. Three putative radical SAM methyltransferases, PctJ, PctN, and PctO, could be responsible for attachment of three carbons on the cyclopentane ring respectively. The generated branched ethyl group is then hydroxylated by a putative P450 monooxygenase PctA and a putative glutathione peroxidase PctB to form a hydroxyethyl group. An amino group of the cyclopentane intermediate could be modified by a putative carbamoyltransferase PctD. The formed carbamoyl group could be then dimethylated by a putative methyltransferase PctF. Although many of the deduced products of the *pct* gene were reasonably assigned, functional characterization of these deduced proteins are indeed necessary to elucidate the complex biosynthetic pathway for pactamycin.

Unassigned *orfs* for the Biosynthesis of Pactamycin

The functions of the deduced products of *pctG*, *pctH*, *pctW*, and *pctX* have not been assigned at the moment. The deduced product of *pctW* shows homology to short-chain dehydrogenase/reductases and thus might be involved in the aforementioned dehydrogenase reactions instead of putative dehydrogenase PctE and PctP. The deduced product of *pctX* shows homology to phosphoglycerate mutases suggesting that PctX might catalyze a phosphatase reaction. However, such phosphate related reaction is unlikely involved in the biosynthesis of pactamycin. Since the direction of translation for the *pctW* and *pctX* gene is opposite to the functionally assigned *orfs* at the upstream, these may be

outside of the *pct* cluster.

The deduced product of *pctH* shows homology to some response regulator proteins with a winged-helix DNA-binding domain in a two-component transcriptional regulatory system. The deduced product of *pctG* contains an *N*-terminal domain with a nucleotide-binding motif (P-loop) and belongs to a P-loop containing ATPase superfamily. Therefore, these continuous gene products could cooperatively control the biosynthesis of pactamycin. The *pctX* gene product may be involved in the transcriptional regulation system by use of a phosphatase activity.

An obvious resistant gene was not found in the *pct* gene cluster. As mentioned above, a pactamycin resistance determinant is encoded in the chromosomal DNA of *S. pactum*. Therefore, the producing microorganism should be resistant to pactamycin despite no resistant gene in the biosynthetic gene cluster. HemK homologous methyltransferase PctF may be involved in the resistant mechanism by modification of ribosomal RNAs.

In conclusion, the pactamycin biosynthetic gene cluster was successfully identified from *S. pactum* NBRC 13433 by use of a gene for radical SAM methyltransferase as a probe. The complex biosynthetic pathway has then been proposed based on the protein sequence of the ORFs encoded in the *pct* cluster. To our knowledge, this work represents the first cloning of a gene cluster for the biosynthesis of cyclopentane containing natural products. The *pctL* gene product was then postulated to catalyze an *N*-glycoside formation between UDP-GlcNAc and 3-aminoacetophenone prior to a unique cyclopentane ring formation. The proposed function of the PctL protein was clearly confirmed with the recombinant enzyme expressed in *E. coli*. This strategy of a glycosylation reaction prior to a unique cyclopentane ring formation could apply for the biosynthesis of the other cyclopentane containing compounds.

Since many of the deduced proteins encoded in the *pct* cluster showed significant homology to the proteins encoded in the gene clusters of secondary metabolites, functional characterization of these, in addition to comparative genetics between the chemical and gene structures, could make a fundamental contribution to natural product chemistry and enzymology. Some specific genes in the *pct* cluster should also be characterized to elucidate the complex pathway for pactamycin. Unprecedented functions of enzymes in nature would be discovered and the findings will lead to identification of novel metabolic pathways.

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