The Journal of Microbiology, March 1997, p.40-46

Vol. 35, No. 1

Nucleotide Sequence Analysis of a Second Set of the Polyketide Synthase β-Ketoacyl synthase and Chain Length Factor Genes from the Salinomycin-producing *Streptomyces albus*

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(Received November 22, 1996 / Accepted December 20, 1996)

The pWHM220 cosmid with a 24-kb insert cloned from *Streptomyces albus* ATCC 21838 induces the biosynthesis of a polyether antibiotic similar to salinomycin in *Streptomyces lividans*. We have analyzed this region by DNA sequencing as well as Southern blot hybridization with type I and type II polyketide synthase (PKS) probes. Surprisingly, we found another set of type II PKS genes only 10-kb from the original PKS genes, *salABCDE*. The DNA sequence revealed two complete open reading frames (ORFs) named *salB2* and *salC2*, and one partial ORF that does not resemble any known DNA or deduced protein sequence. The *salC2* should code for chain length determining factor while the deduced amino acid sequence encoded by *salB2* exhibits high similarity to β-ketoacyl synthase from different PKS gene clusters. The highest identity was found for β-ketoacyl synthases from *S. argillaceus* (MtmP, 59.1% identity), the mithramycin producer and from *S. venezuelae* ISP5230 (JadA, 52.3% identity), the jadomycin producer. The SalC2 protein clearly resembles its counterparts in other aromatic PKS gene clusters that are believed to influence the length of the polyketide chain. The highest identities observed were to that of *S. argillaceus* (MtmK, 62.3%) and *S. venezuelae* ISP5230 (JadB, 55.1%) proteins. Moreover, the deduced amino acid sequences of the *salB2* and *salC2* products were 29.0% identical.

Key words: *Streptomyces albus*, β-ketoacyl synthase, chain length factor

Polyketides are a large group of structurally diverse secondary metabolites, several of which have applications as antibiotics, immunosuppressants, anticancer agents, and veterinary products. In recent years genetic studies have yielded detailed information on the organization and function of genes involved in the biosynthesis of polyketides in microorganism (16, 17).

Cloning, sequencing, and functional analysis of these genes have improved our understanding of the genetic programming of polyketide synthases (16, 24, 25, 26). Two classes of PKSs have been studied from microorganisms (type I and type II (Fig. 1)). Various examples of the type II PKS have been identified (3, 5, 7, 8, 9, 12, 14, 15, 21, 22, 32, 35), which consists of a multienzyme complex formed by several polypeptides with individual enzymatic activities. In contrast, there is a paucity of information about type I PKS where the enzyamtic activities are present as separate domains in one multifunctional polypeptide. This is the case for the PKS involved in biosynthesis of the macrolide antibiotics, such as erythromycin (11) and rapamycin (30).

All of these metabolites share a common mechanism of biosynthesis that has largely resisted in vivo analysis. The carbon skeleton of a polyketide results from the sequential condensation of fatty acids like acetate, propionate, and butyrate. This process is catalyzed by polyketide syhthases in a manner that is conceptually similar to the biosynthesis of long-chain fatty acids catalyzed by the fatty acids synthases found in all organisms (17).

We reported recently the type II PKS gene cluster from the salinomycin producer (8). Five of the genes cloned in pWHM220 were characterized by sequence analysis to confirm that the 3.9-kb DNA fragment contained type II PKS homologs (Fig. 2). The five open reading frames were identified by sequence analysis and named *salA-salE*. Since *salA*, *salB*, *salC*, *salD*, and *salE* genes encode proteins

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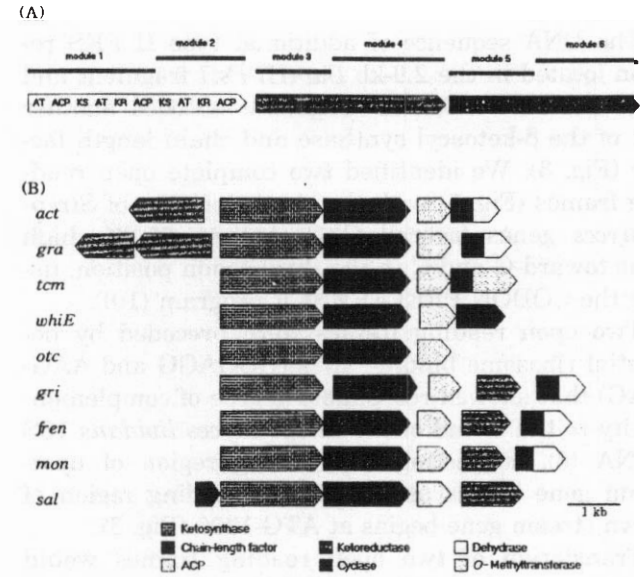

Fig. 1. Comparison of the organization between type I and type II PKSs. (a) Type I PKS for erythromycin biosynthesis. (b) The known gene clusters encoding type II PKSs. References: *act* (12), *fren* (5), *gra* (33), *gri* (35), *mon* (3), *otc* (21), *sal* (8), *tcm* (7), *whiE* (9).

highly similar to the following enzymes : cyclase, β-ketoacyl synthase, chain length determining factor, acyl carrier protein, and ketoreductase; it is very likely that these five *S. albus* genes govern the synthesis of some type of aromatic polyketide.

In this paper we report the additional PKS genes which was found by further sequencing of pWHM 220. These new genes, named *salB2* and *salC2* showed homology with *tcmKL*(7). The two genes seems to encode β-ketoacyl synthase and chain length factor homologs, compose of minimal PKS together with acyl carrier protein.

Materials and Methods

Bacterial strains, bacteriophage, and plasmids

S. albus ATCC21838 was obtained from the American Type Culture Collection (Rockville, Md). *Escharichia coli* DH5α was purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md) and was used for subcloning and squencing. *E. coli* vector used for subcloning was pUC19 (GIBCO-BRL, Gaithersburg Md) and for sequencing, M13mp18 and M13mp19 were used (33). The cosmid clone pWHM220 has been described elsewhere (8). The cosmid vector pKC505 (27) was obtained from Richard Baltz, Eli Lilly Co. Ltd. (Indianapolis, Ind.).

Media, enzymes, and chemicals

Bacterial strains were grown on LB medium where 50 μg/ml ampicillin was added, when necessary. Restriction enzymes, ligase, and klenow enzyme were purchased from Takara Co. All enzyme reactions were carried out according to the recommendations of manufacturers, unless indicated otherwise. All chemicals were purchased from Sigma Chemical Co. (U.S.A).

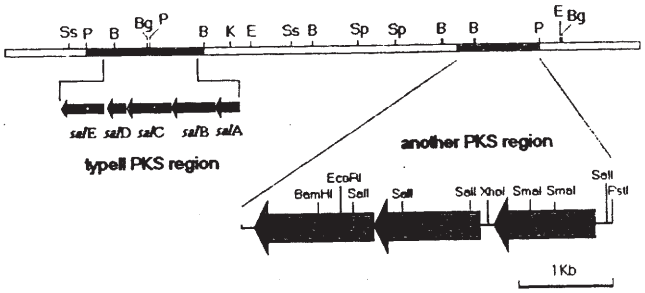

Fig. 2. Restriction map of 24-kb region of *S. albus* ATCC 21838 genomic DNA cloned in pWHM220.

DNA manipulation

The general DNA manipulation was carried out following Sambrock *et al* (28). Plasmid DNA and bacteriophage RF DNA were isolated from *E. coli* by using alkaline SDS extraction of cell lysate. A large-scale DNA purificaton was performed by banding in CsCl-ethidium bromide density gradients. Single-stranded DNA was isolated by precipitating phage particles. Agarose gel electrophoresis was performed in Tris-acetate or Tris-borate buffer.

Nucleotide sequencing and analysis of sequence

Nested deletions were constructed with the Erase a Base system (Promega Biotech) according to the manufacturer's instruction. Derivatives of pUC19 and M13mp19 containing *S. albus* DNA were digested with *Xba*I-*Pst*I and *Bam*HI-*Kpn*I, respectively before exonuclease III treatment.

Double-stranded templates were subcloned into M13mp18 and single-stranded templetes were sequenced by the dideoxy chain-termination method using (α-^{32}P)dATP and Sequenase version 2.0 (United States Biochemicals) according to the manufacturer's instructions. To avoid compressions, 7-deaza-dGTP was used instead of dGTP. Labeled DNA was separated on 6% polyacrylamide wedge gels.

Primary DNA sequence data were analyzed and assembled by using software from DNASIS and BLAST programs (1,13). DNA and protein sequence were analyzed with the Genetics Computer Group software package (10).

Results and Discussion

Southern analysis of pWHM220 and isolation

of DNA homologous to *tcmKL*

The *actI* gene encoding a type II PKS involved in the biosynthesis of actinorhodin (12), an aromatic polyketide which has played a key role in the elucidation of polyketide synthesis mechanism and β-ketoacyl synthase domain of the *eryA* type I PKS gene(11) were used for Southern analysis of cosmid pWHM220 (Fig. 2). This established the presence of 1.4-kb, 2.4-kb, and 2.9-kb *Bam*HI-*Pst*I fragments that hybridized to *actI* probe, but very faintly to *ery*KS. Because previously described (8) type II polyketide synthase genes from *S. albus* were found in the 3.8-kb *Bam*HI fragment that contains one *Pst*I site and yields 1.4-kb and 2.4-kb *Bam*HI-*Pst*I fragments, we concluded that the 2.9-kb *Bam*HI-*Pst*I fragment should be located in different place from the 3.8-kb *Bam*HI fragment.

Sequence analysis of another PKS genes in pWHM220

The DNA sequence of additional type II PKS region located in the 2.9-kb *Bam*HI-*Pst*I fragment and adjacent 1.3-kb *Bam*HI fragment, encodes another set of the β-ketoacyl synthase and chain length factor (Fig. 3). We identified two complete open reading frames (Fig. 5) with the characteristics of *Streptomyces* genes (overall G+C content, 75.2%; high bias toward G and C in the third codon position, using the CODON PREFERENCE program (10)).

Two open reading frames were preceded by potential ribosome binding sites (GGGAGG and AAGGAG) that showed reasonable degree of complementarity to the 3' end of the *Streptomyces lividans* 16S rRNA (6), suggesting that coding region of upstream gene begins at GTG-71 and coding region of down stream gene begins at ATG-1300 (Fig. 3).

Translation of two open reading frames would result in a polypeptide with 411 amino acids and

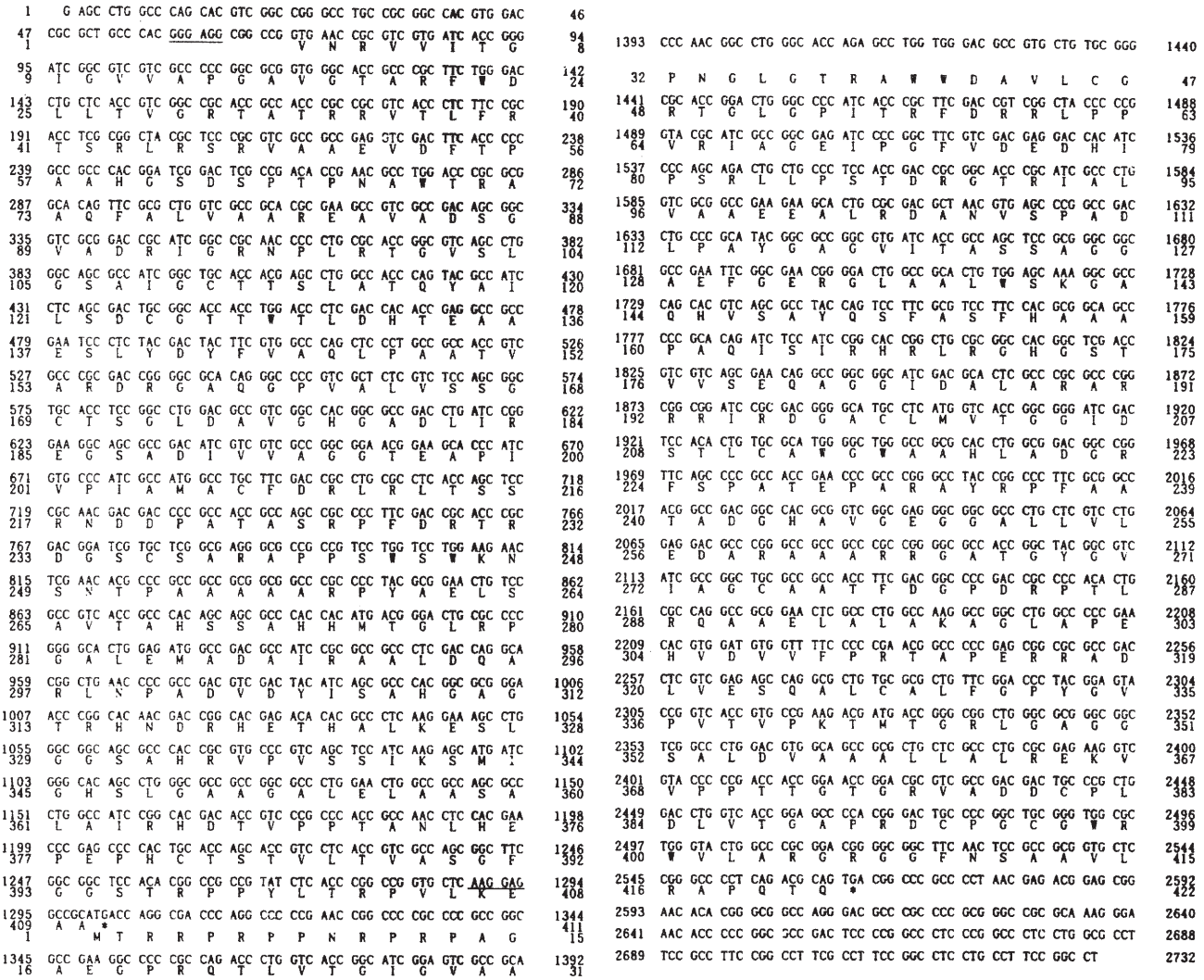

Fig. 3. Nucleotide sequence of a 2,732-bp region encoding *salB2* and *salC2*. The predicted peptide sequence is shown below the nucleotide sequence. Putative ribosome binding sites are underlined.

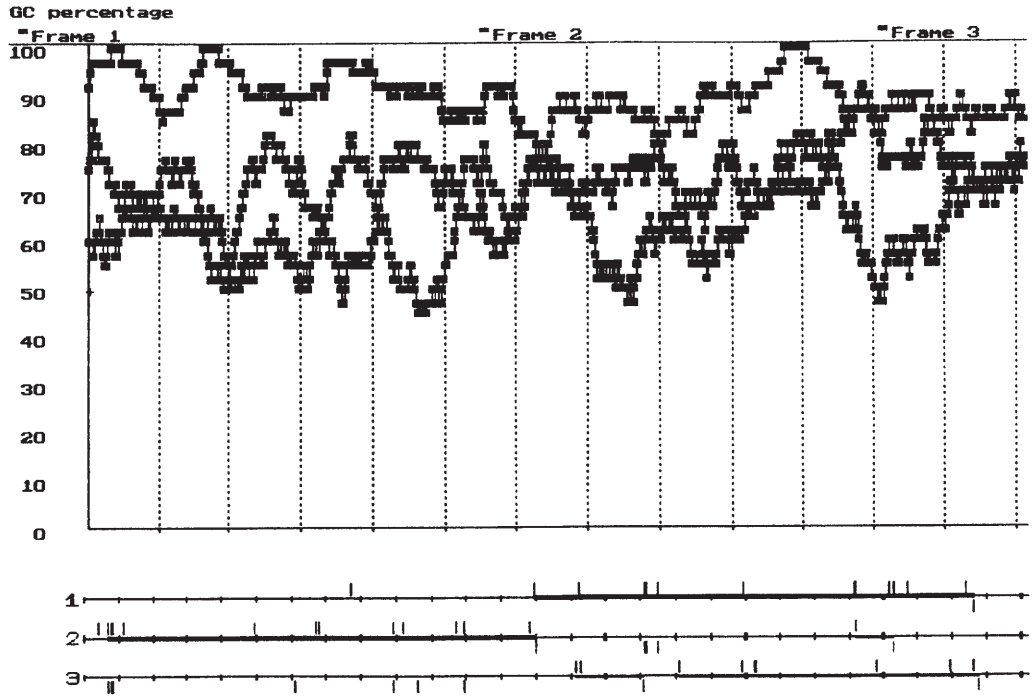

Fig. 4. Analysis of guanine and cytosine content at third positions, for 2,732-bp with the *salB2* and *salC2* genes of pWHM220. ATG and GTG codons are indicated by vertical bars, stop codons are designated by crosses.

422 amino acids, respectively, and a molecular weight of 43,621 and 43,738, and a calculated isoelectric point of 7.72 and 9.60, respectively. The gene organization for the two genes seemed to be translationally coupled.

Deduced function of the proteins

The two new genes cloned in pWHM220 were proved to have homology with *tcmKL* by sequence analysis. TcmK and TcmL compose minimal PKS for tetracenomycin biosynthesis (7). The separate components of the minimal PKS are ketoacyl synthase (KS) which catalyzes the condensation reaction and may also carry an acyltransferase (AT) domain for loading the starter unit to the KS, a second protein with a high degree of homology to the KS which is involved, at least in part, in determination of the polyketide chain length, and an acyl carrier protein (ACP).

The two open reading frames were identified by CODONPREFERENCE and named *salB2, salC2*. Sequence comparisons with GAP program (10) indicated that SalB2 polypeptide bears strong similarty to many other β-ketoacyl synthases. The similarity is especially strong in the region surrounding 169-Cys and 347-Ser, which corresponds to the

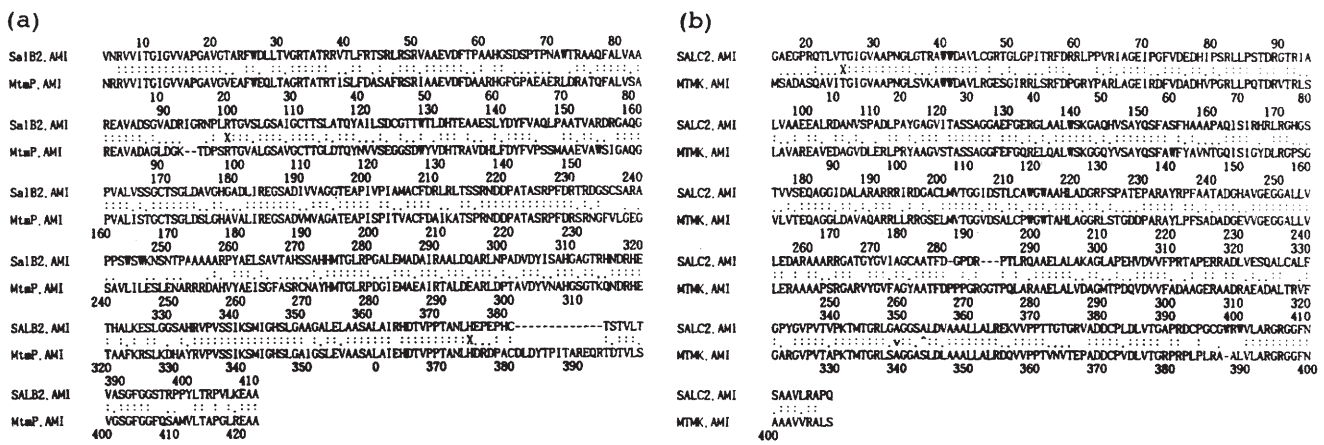

Fig. 5. Amino acid sequence comparisons using Genetic Computer Group program. (a) SalB2 and MtmP, β-ketoacyl synthase for mithramycin biosynthesis (59.1% identity); (b) SalC2 and MtmK, chain length factor from *S. argillaceus* (62.3% identity).

A. β-ketoacyl synthase active site

		Ref
S. albus SalB2	RGAQGPVALVSSGTSGLDAVGHGA	this study
S. argillaceus MtmP	IGAQGPVALISTGTSGLDSLGHAV	22
S. venezuelae JadA	VGAEGPNTVVSTGTSGLDSVGYAR	15
S. glaucescens TcmK	AGAEGPVTVVSTGTSGLDAVGYGT	7
S. griseus Gri	VGAEGPATVVSTGTAGIDAVGHAV	35
S rimosus Otc	AEAEGPAGVVSAGTSGIDVLTHAA	21
S. violaceoruber Gra	AGAEGPVTMVSDGTSGLDSVGYAV	32
S. peucetius Dau	AGAEGPVNIVSAGTSGIDSIGYAC	14
S. coelicolor Act	VGAEGPVTMVSTGTSGLDSVGNAV	12
S. albus SalB	VGAEGPSTVVSTGTSGLDSVGYAV	8
Active site system	*A*GP *S G T*G ** *	

B. Acyltransferase active site

		Ref
S. albus SalB2	PVSSIKSMIGHSLGAAGALELAASAL	this study
S. argillaceus MtmP	PVSSIKSMIGHSLGAIGSLEVAASAL	22
S. venezuelae JadB	PVSSIKSMVGHSLGAIGSIEIAASAL	15
S. glaucescens TcmK	PVSSIKSMIGHSLGAIGSLELAACAL	7
S. griseus Gri	PVSAIKSMVGHSLGAIGSIEIAACAL	35
S rimosus Otc	PISSIKSMIGHSLGAICALEVAASAL	21
S. violaceoruber Gra	PVSSIKSMGGHSLGAIGSIEIAASVL	32
S. peucetius Dau	PISSIKSMIGHSLGAVGSLEVAATAL	14
S. coelicolor Act	PVSSIKSMVGHSLGAIGSLEIAACVL	12
S. albus SalB	PVSSIKSMVGHSLGAIGSIEIAASAL	8
Active site system	P*S**KSM GH*SLGA **E*AA* L	

Fig. 6. Sequence alignment of amino acids of β-ketoacyl synthase (KS) and acyltransferase (AT) with conserved active sites of other KS and AT, respectively. Conserved amino acids for all peptides are shown under each alignment. Amino acids at active sites are marked in box. References for each peptide are shown.

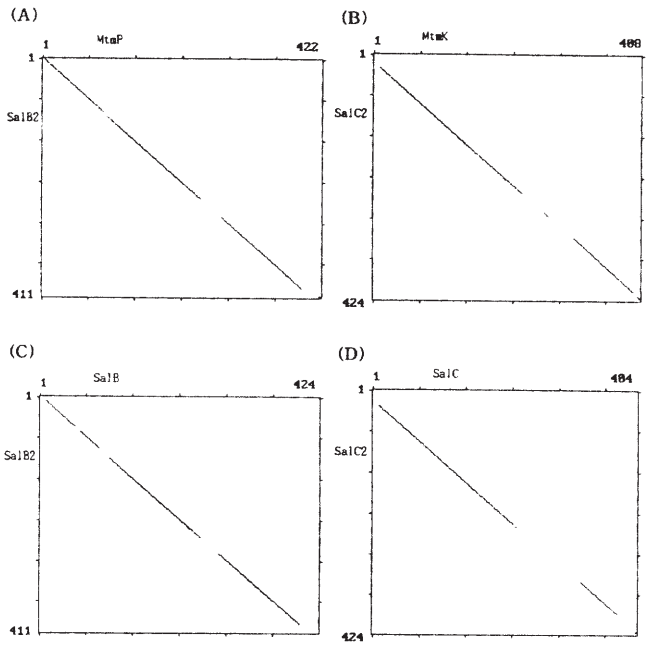

Fig. 7. COMPARE-DOTPLOT analysis of the deduced protein products of (A) SalB2 versus MtmP, 59.1% identity; (B) SalC2 versus MtmK, 62.3% identity ; (C) SalB2 versus SalB, 54.9% identity; (D) SalC2 versus SalC, 54.8% identity. A window size of 30 was used at a stringency of 15.

active site in β-ketoacyl synthase enzyme (Fig. 6).

The deduced *salC2* product resembles several

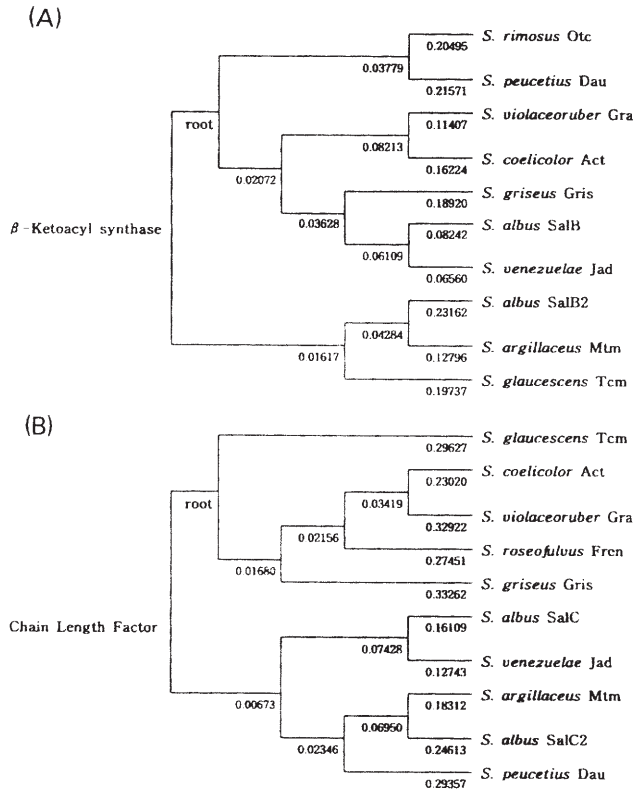

Fig. 8. Dendrogram showing relationahips of type II PKS components, β-ketoacyl synthase and chain length determining factor. PILEUP program of GCG (10) was used to analyze the data.

chain length factor with the following percentages of identical amino acids (Fig. 4, 7): MtmK for mithramycin biosynthesis, 62.3% (22), JadB for jadomycin biosynthesis, 55.1% (15), and SalC for polyketide biosynthesis in *S. albus*, 54.8% (8).

All of the genes are transcribed in the same direction. A phylogenetic tree, involving a large set of sequence including those of *salB2* and *salC2*, is shown in Fig 8. Recent studies have shown that combination of genes from different PKSs can be expressed to produce functional hybrid PKS. The PKS gene set identified in this study is an important addition to the range of available PKSs and will enable further analysis of the molecular basis for PKS programming to be made.

Relationship between the two polyketide gene clusters

We had previously shown that *S. albus* has a gene cluster (*salA-E*) encoding type II PKS. And this region could complement mutations in the *tcmKL* region (8). In this study, we showed that pWHM220 cosmid clone had another copy of genes for β-ketosynthase and chain length factor. The presence of multiple copies of the same or similar genes in

Streptomyces is not usual but not odd(11, 23).

Here we report a second set of PKS genes (*salB2, salC2*) in *S. albus* that are similar to another set, the *salBC* genes from another PKS gene cluster (8); *salB2* encodes the β-ketoacyl synthase and *salC2* encodes chain length factor in the same orientation. Since the original PKS gene cluster and the additional PKS gene cluster are close each other (only 10-kb apart) and have the same orientation, we speculate that these two gene clusters might have arisen by tandem genetic duplication (2).

However, we do not exclude that the two PKS gene clusters have different functions. Since *act*- and *whiE*-PKS genes, the two sets of related genes, are expressed at different stages in the life-cycle of the organism, *S. coelicolor,* to produce antibiotic and spore pigments, respectively. It was found that each of the three subunits of the *whiE* minimal PKS could complement lesions in the *act*-PKS to produce actinorhodin. Conversely, the corresponding *act*-PKS subunits would complement mutations in the *whiE* locus to restore spore pigmentation (34).

Acknowledgments

This work was supported by a grant of Genetic Engineering Research Program of the Ministry of Education, Korea, in 1995.

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