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Genetic analysis of polyketide biosynthetic genes isolated from *Streptomyces albus*, a salinomycin producer.

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Sequence analysis of a DNA region encompassing the site of hybridization to *actI*, the gene for type II minimal polyketide synthase (PKS) for actinorhodin biosynthesis, from *Streptomyces ablus* revealed three more complete open reading frames additional to the already found two genes, plausibly encoding β-ketoacyl synthase/acyl transferase (KS/AT) and chain length determining factor (CLF). The open reading frames (ORFs) were named *salA*, *salD*, and *salE*, from the upstream. In the homology analysis of the deduced amino acid sequences, *SalA* resembles the *Streptomyces glaucescens* TcmI, decaketide cyclase, SalD resembles acyl carrier protein in type II PKS, and SalE resembles the ActIII ketoreductase. The whole 4.4 kb of DNA sequence obeys the same conservation pattern as other type II PKSs. Therefore, we suggest that the 4.4 kb DNA from *Streptomyces albus* encompasses genes encoding enzymes for polyketide biogenesis in the organism and its organization is type II. The exsitence of SalA, an analogue of the aromatic cyclase, revealed a relatedness of the 4.4 kb DNA with the aromatic PKS.

Microorganisms and plants produce a collection of metabolites called polyketides. Polyketides are formed by iterated condensation of simple fatty acid-thio ester derivatives. Many of the polyketide metabolites have specific physiological roles in the producer organisms, some with important applications as antibiotics or chemotherapeutic agents. Many polyketide-derived antibiotics are produced by *Streptomyces*. *Streptomyces* is mycerial, gram-positive, aerobic soil bacteria and the genetics of antibiotic biosynthesis systems in *Streptomyces* have attracted research interests due to their commercial usefulness.

A common feature among macrolide PKS (polyketide synthase) genes is that they encode large multifunctional polypeptides containing putative FAS (Fatty Acid Synthase)-like activities, generally arranged in the same relative order as that found in type I animal FASs and fungal PKSs (7, 23). This type of organization differs substantially from the type II PKS system for aromatic polyketides, in which monofunctional polypetides are assembled by non-covalent bonding to multifunctional complex (17, 30). In addition, all the type I PKS genes thus far characterized consist of repeated units designated *modules* (8, 9, 19).

The actinorhodin biosynthetic gene (*act*) in *Streptomyces coelicolor* has been extensively studied and is a representative type II PKS gene (1, 6, 10, 13). With southern hybridization analysis, many streptomycetes are shown to have the region homologous to *actI*, the gene encodes enzymes acting in the early reaction in polyketide biogenesis and many aromatic PKS genes are cloned by using *actI* as a hybridization probe (3, 22). To date, many PKS genes have been cloned and their nucleotide sequences determined (19), some cases of hybrid antibiotics production using DNA recombinat technology have been reported (14, 27). It was found that *actI* homologous regions are highly conserved in their location, total length, and predicted amino acid sequence (10, 18, 32). There are genes for KS/AT (β-ketoacyl synthase and acyl transferase), CLF (Chain Length Determining Factor) and ACP (Acyl Carrier Protein) in *actI* homlogous regions and their products are collectively called "minimal PKSs" (25).

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An about 24 kb DNA was isolated using the *actI* as a heterologous hybridization probe from the pKC 505 genomic library of *Streptomyces albus* ATCC 21838 (Materials and Methods). The pKC 505 clone encompassing the 24 kb *Streptomyces albus* DNA was named pWHM 210 (21). Sequence analysis of the 3.8 kb *Bam*H I region in the 24 kb DNA that encompassed the site of hybridization to *actI* revealed sequences encoding KS/AT (β-ketoacyl synthase & acyl transferase) and CLF (chain length determining factor). The results were reported elsewhere (21) and here, we report other genes revealed in this region.

MATERIALS AND METHODS

Bacterials and Plasmids

The genomic library of *Streptomyces albus* ATCC 2 1838 was prepared using *Escherichia-Streptomyces* shuttle cosmid vector pKC 505 (Eli Lilly). *Streptomyces albus* chromosomal DNA was partially digested with *Mbo*I and ligated to unique *Bam*HI site of pKC 505. The DNA restriction fragments of pWHM 210 were subcloned using pGEM-3zf(-) (Promega) as a vector. M13mp19 (New England Biolabs) was used for the preparation of sequencing templates.

E. coli JM109(*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* △[*lac-proAB*], [F′, *traD36, proAB, lacI*^{q} *Z*△*M15*] was used to propagate recombinant pGEM-3zf(-). *E. coli* DH5α F′^{TM} (F′, ϕ80 d*lacZ*△*M15,* △[*lac-ZYA-argF*], U169, *recA1, endA1, hsdR17*[rk$^-$, mk$^+$], *supE44,* λ$^-$, *thi-1, gyrA, relA1*) was used as a recipient for recombinant M13mp19 vectors.

Media

E. coli cells were cultured in LB medium (1% Bactotryptone [Difco], 0.5% yeast extract, 1% NaCl, pH 7.5). *E. coli* transformants were selected on LB agar broth (1.5% Bacto-agar) and 3ml of LB top agar (0.75% Bacto-agar) containing 20μl of 2% 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), 10μl of 20% isopropyl-β-D-thiogalactoside (IPTG), and 200μl of the host culture suspension.

Enzymes and Reagents

X-gal and IPTG were purchased from Sigma Co. All restriction endonucleases and T4 DNA ligase were purchased from Pharmacia, Boehringer Mannheim, KOSCO and BRL. Sequenase^{TM} version 2.0 kit for DNA sequencing was from United States Biochemical, and Erase a Base kit for nested deletion was purchased from Promega. [α-^{32}P] dATP, [α-^{32}P] dCTP, [α-^{35}S] dCTP, Hybond-N memebrane and ECL gene detection system RPN 210 1-version 2 were purchased from Amersham. All the materials were used as recommended by the suppliers.

DNA Isolation

Chromosomal DNA was extracted from *Streptomyces albus* by the method of Hopwood et al (15). 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 purification was performed by banding in CsCl-ethidium bromide density gradients. Single-stranded DNA was isolated by precipitating phage particles with PEG. DNAs were resolved by doing horizontal agarose gel electrophoresis in Tris-acetate or Tris-borate buffer.

Preparation of Sequencing Templates

The 3.8 kb *Bam*HI fragment from pWHM210 was subcloned in the *Bam*HI site of pGEM-3zf(-). The 1.4 kb of *Sal*I fragment from the 3.8 kb of DNA was subcloned in the *Sal*I site of M13mp19 in both orientations relative to *lac* promoter. The 1.2 kb *Sal*I fragment generated with *Sal*I site in the 3.8 kb DNA and *Sal*Isite in polycloning site in pGEM-3zf(-) was also subcloned in the *Sal*I site of M13mp19 in both orientations relative to *lac* promoter. 3 out of the 4 recombinant M13mp19 were deleted unidirectionally using Erase a Base kit with *Bam*HI and *Sac*I digestion. The remaining one was not able to be deleted because there was no appropriate restriction sites (*Bam*HI cut liberated insert DNA). The restriction sites (*Sma*I, *Pvu*II, *Nco*I, *Xho*I) in the 2.6 kb of DNA (the 1.2 kb of *Sal*I fragment plus the 1.4 kb of *Sal*I fragment) were used for generating sequencing templates. Deletion clones of pGEM-3zf(-) were sequenced for connecting two *Sal*I fragments. Sequencing with synthetic deoxyoligonucleotide was performed in highly compressed regions to confirm the sequences.

The nucleotide sequences of the remaining region in the 3.8 kb of DNA and an about 600 bp adjascent fragment downstream were determined using synthetic deoxyoligonucleotide.

Nucleoide Sequencing and Analysis

Fragments of appropriate size in the M13 deletion subclone were sequenced by the dideoxy chain termination method of Sanger et al (29). DNA chain termination sequencing with Sequenase^{TM}enzyme were performed according to the protocols given by the supplier. Electrophoresis was carried out on an 8% polyacrylamide/8 M urea gel. Sequence data were analysed by the FRAME (4), DNASIS (Parmacia), and PROSIS (Parmacia). Due to problems with the secondary structure when sequencing DNA with high G+C content, sequencing was carried out using nucleotide analogues (dITP or 7-deaza dGTP).

RESULT AND DISCUSSION

To ask whether the polyether antibiotic salinomycin

(sal) was assembled by a type I or a type II polyketide synthase in *Streptomyces albus*, clones that hybridized to *actI* and *actIII* genes were isolated from a cosmid library of ATCC 21838 DNA. *actI* and *actIII* were genes for KS/AT and CLF of actinorhodin biosynthesis in *Streptomyces coelicolor*, and used as heterologous hybridization probe. *actI* (about 4 kb) and *actIII* (about 0.8 kb) probes were subcloned in the *Bam*HI site of pBR329, and the probe was made using nick translation system (21). Clone pWHM 210 that contained an approximately 24 kb DNA insert was isolated. 3.8 kb of *Bam*HI fragment in pWHM 210 was hybridized to *actI*. The *actI* hybridization region was presented in Fig. 1.

The nucleotide sequence of the 4.4 kb region, encompassing the 3.8 kb DNA, was determined by the strategy outlined in Fig. 1. We reported the existence of KS/AT and CLF genes in the 3.8 kb *Bam*HI fragment of pWHM 210 (21). The fact that genes for KS/AT and CLF were found as monofunctional polypetides (different translation units) revealed that its organization was type II (21). Because the biosynthesis genes have been proven to have clustered organization in the type II PKS, it is certain that other genes for polyketide biosynthesis locate in or near by the cloned region.

The sequence of the remaining region was analyzed for probable protein coding regions using the CODON-REFERENCE program (4, 5). The sequences for 16S rRNA binding were found in the probable locations (2). The analysis revealed the presence of two more complete ORFs and a N-terminal region of phanthom gene. Thus, the downstream region adjacent to the 3.8 kb *Bam*HI fragment was subcloned and the sequence of one more complete ORF was found. The nucleotide sequences of the ORFs are presented in Fig. 2. Each ORF is preceded by a probable ribosomal binding site, a sequence showing some similarity to the 3' end of the *Streptomyces coelicolor* 16S rRNA (2), and named *salA, D,* and *E* from the upstream. The gene organization

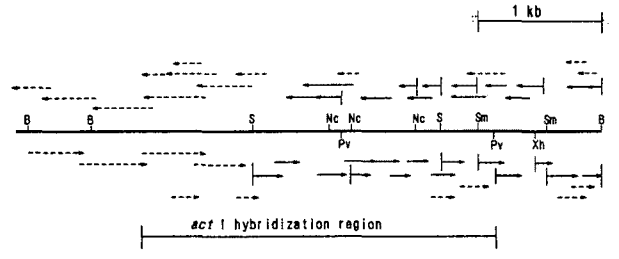

Fig. 1. Restriction map and sequencing strategy of the 4.4 kb DNA fragement from pWHM210.

Clones used for sequencing were obtained as described in Materials and Methods. The arrow indicate the extent of sequence obtained from each clones (The dashed arrow indicate the extent of sequence obtained using synthetic oligonucleotide as sequencing primer). B, Nc, Pv, S, Sm, and Xh indicate *Bam*HI, *Nco*I, *Pvu*I, *Sal*I, *Sma*I, and *Xho*I, respectively.

of the 4.4 kb DNA encompassing *salA,B,C,D,* and *E* is shown in Fig. 3. *salA, D,* and *E* were preceded by the sequence of AGGAGC from -14 position, AGGACC from -10 position, and AGGAGC from -12 position, respectively. Here, -1 means 1 base pair upstream to the start codon, ATG. The predicted amino acid sequences showed similarities to those of *tcmI, tcmM* and *actIII*, respectively in Homology Search program in PROSIS (Pharmacia).

tcmI analogues catalyzed the cyclization reaction found in a decaketide biosynthetic gene cluster, such

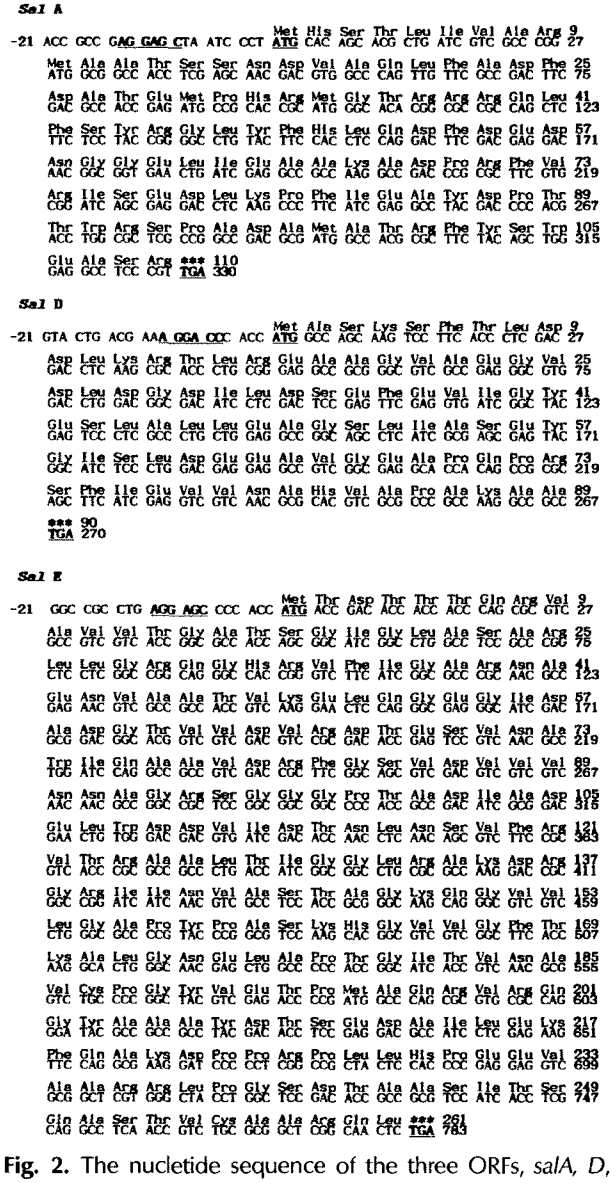

Fig. 2. The nucleotide sequence of the three ORFs, *salA, D,* and *E*.

The translational start and stop sites are in bold face and underlined. The potential ribosomal binding sites preceding the ORFs are underlined, also. The predicted amino acid sequence was shown above the nucleotide sequence.

as *tcm*. Tcm F2 cyclase, the product of *tcmI*, was postulated to mediate intramolecular aldol or Claisen condensation, and the following dehydration, at the site of C-2 and C-9 (18). Since different kinds of cyclized compounds such as aklaviketone in *Streptomyces peucetius*, dehydrorabelomycin in *Streptomyces murayamaensis*, and tetracenomycin F2 in *S. glaucescens*, formally can be derived from the same class of oligoketide intermediate, the cyclase could be an important determinant of the structural variation among aromatic decaketides (18, 31).

tcmM homologues are genes for acyl carrier protein (ACP) of type II PKS, universally found adjacent to that for CLF. *ActIII* mediates C-9 ketoreduction in actinorhodin biosynthesis and is not essential for polyketide biosynthesis. The identity between SalA, D, E and TcmI, M, & ActIII in the Homology Search program (PROSIS) were 35.9%, 33.3%, and 55.2%, respectively. Homology Plot diagram (PROSIS) of SalA, D, E versus TcmI, M, & ActIII are shown in Fig. 4.

The whole 4.4 kb of DNA sequence obeys the same conservation pattern as other type II PKSs (Fig. 5). Thus, we suggest that the 4.4 kb DNA from *Streptomyces albus* encompasses genes encoding enzymes for the polyketide biogenesis in the organism and its organization is type II. *salD*, the presumptive ACP gene in type II PKS in *Streptomyces albus*, is located adjacent to *salBC*, the gene for KS/AT and CLF. The products of *salBCD* were plausible to be minimal PKS of type II system in *Streptomyces albus*. Alignments of the putative active site of *salD* with ACP regions from different origins was shown in Fig. 6.

The presence of *salA*, the gene for enzyme catalyzing adol type intramolecular cyclization presents the possibility that the 4.4 kb gene is the one responsible for aromatic polyketide biosynthesis contrary to the result of preceding experiments such as transformation to heterologous host, *Streptomyces lividans* (21). In the preceding experiment, *actI* probe hybridized to a single DNA fragment from *Streptomyces albus*, it was thought that the hybridized DNA region would be responsible for salinomycin biosynthesis, the major polyketide pro-

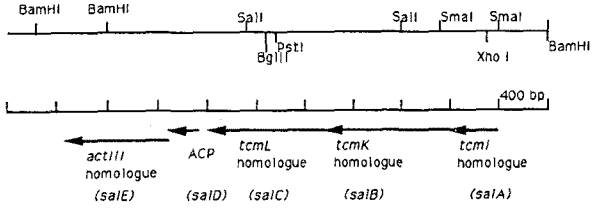

Fig. 3. The gene organization found in the 4.4 kb region from *Streptomyces albus* (21)

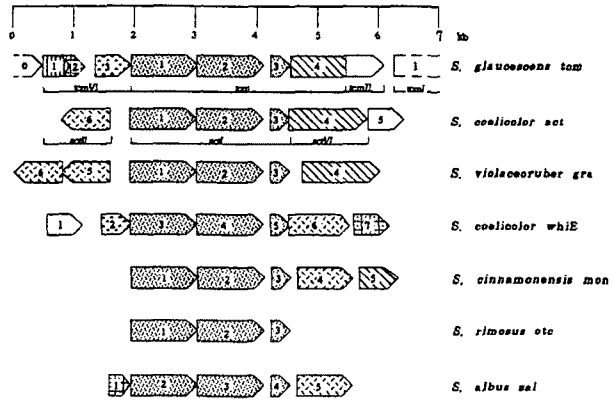

Fig. 5. Comparison of genetic structure, direction of transcription and function among different type II PKSs.
Genes predicted to have a similar function have identical shading. The numbering is arbitrary. tcm; tetracenomycin, Streptomyces glaucescens (5). gra; granaticin, *Streptomyces violaceoruber* (32), *act*; actinorhodin, *Streptomyces coelicolor whiE*; presumptive spore pigment, *Streptomyces coelicolor*, *otc*; oxytetracyclin, *Streptomyces rimosus*, monensin, *Sterptomyces cinnamonensis* (18). *sal*; salinomycin, *Streptomyces albus* (21 and this work)

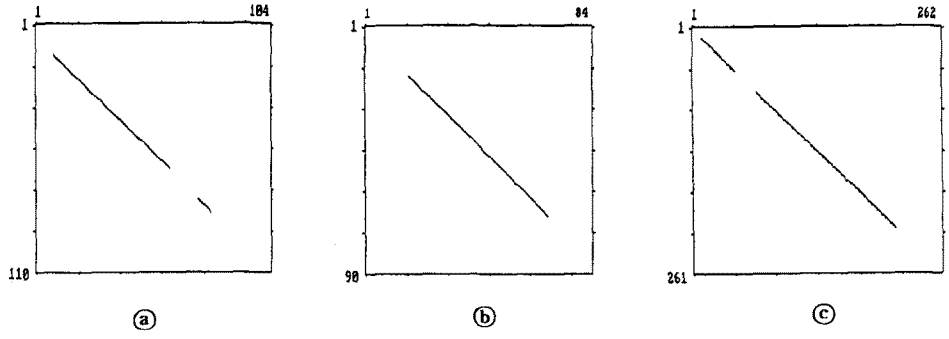

Fig. 4. Homology Plot of the predicted protein product (window size, a stringency)
The nucleotide sequences of *tcmI, J* and *actIII* were obtaied from EMBL library with Database Access of DNASIS. [a] SalA versus TcmI, decaketide cyclase of tetracenomycin biosynthesis in *Streptomyces glaucescens* (30, 15). [b] SalD versus TcmM, ACP in tetracenomycin biosynthesis in *Streptomyces glaucescens* (30, 17) [c] SalE versus ActIII, ketoreductase in actinorhodin biosynthesis in *Streptomyces coelicolor* (30, 20).

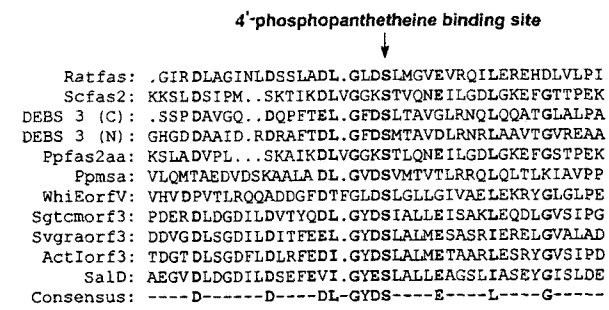

Fig. 6. Alignments of the putative active sites for the SalD with ACP regions of other FASs and PKSs from different origins (10); a derived consensus sequence is given below.

The conserved residues are in boldface. *Ratfas,* rat FAS; Scfas2, *S. cerevisiae* FAS2; Ppfas2, *P. patulum* MSA synthase; DEBS 3 (C, N), *S. erythraea* EryA-ORFA, domains C and N; Sgtcmorf3, *S. glaucescens* tcmI-ORF3; Svgraorf3, *S. violaceoruber* Gra-ORF3; *S. coelicolor* ActI-ORF3; *SalD, S. albus salD (this work).*

duct in this organism. The functions and the substrate specificities could not be confirmed absolutely from such a simple comparison of the gene products, and moreover, the genetics and the enzymology of a polyether biogenesis are still completely unrevealed territories.

The characterization of regions adjacent to the 4.4 kb DNA in pWHM 210 would reveal other genes for polyketide biosynthesis. If this cloned DNA is related to an aromatic polyketide, other genes for cyclization and dehydration, analogues of *actVII, IV* or *tcmJ* are plausibly located near this region (10, 19, 26, 33). Gene disruption experiments as well as 'chimeric PKS' (11, 12, 24, 25, 26, 32) would be helpful to confirm the fuctions. Further studies were carried out to confirm the functions in gene disruption experiments, but the efforts failed due to the instability of salinomycin production which is probably related with chromosomal DNA rearrangement. The full sequencing of the 24 kb insert of the pWHM 210 has been carried on in our labaratory, and a more delicate approach such as 'chimeric PKS' will be tried if the expression system (25) is attainable in the near furture.

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