Phenolic Lipids Synthesized by Type III Polyketide Synthase Confer Penicillin Resistance on Streptomyces griseus

Masanori Funabashi, Nobutaka Funa, and Sueharu Horinouchi

From the Department of Biotechnology, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Type III polyketide synthases (PKSs) found in plants, fungi, and bacteria synthesize a variety of aromatic polyketides. A Gram-positive, filamentous bacterium *Streptomyces griseus* contained an *srs* operon, in which *srsA* encoded a type III PKS, *srsB* encoded a methyltransferase, and *srsC* encoded a flavoprotein hydroxylase. Consistent with this annotation, overexpression of the *srs* genes in a heterologous host, *Streptomyces lividans*, showed that *SrsA* was a type III PKS responsible for synthesis of phenolic lipids, alkylresorcinols, and alkylpyrones. *SrsB* was a methyltransferase acting on the phenolic lipids to yield alkylresorcinol methyl ethers, and *SrsC* was a hydroxylase acting on the alkylresorcinol methyl ethers. *In vitro* *SrsA* reaction showed that *SrsA* synthesized alkylresorcinols from acyl-CoAs of various chain lengths as a starter substrate, one molecule of methylmalonyl-CoA, and two molecules of malonyl-CoA. *SrsA* was thus unique in that it incorporated the extender substrates in a strictly controlled order of malonyl-CoA, malonyl-CoA, and methylmalonyl-CoA to produce alkylresorcinols. An *srsA* mutant, which produced no phenolic lipids, was highly sensitive to β-lactam antibiotics, such as penicillin G and cephalaxin. Together with the fact that the alkylresorcinols were fractionated mainly in the cell wall fraction, this observation suggests that the phenolic lipids, perhaps associated with the cytoplasmic membrane because of their amphiphilic property, affect the characteristic and rigidity of the cytoplasmic membrane/peptidoglycan of a variety of bacteria. An *srs*-like operon is found widely among Gram-positive and -negative bacteria, indicating wide distribution of the phenolic lipids.

Type III PKSs are structurally and functionally simple PKSs that catalyze the synthesis of aromatic polyketides in both plants and microorganisms (1). We previously found that a type III PKS, *RppA*, in the Gram-positive, filamentous bacterium *Streptomyces griseus* catalyzes the synthesis of 1,3,6,8-tetrahydroxynaphthalene by using malonyl-CoA as a starter, carrying out four successive extensions with malonyl-CoA and cyclizing the resulting pentaketide to the naphthalene scaffold (2). This was the first report of a functional type III PKS from bacteria. Since then, genome projects of bacteria have predicted that type III PKSs are distributed widely not only in *Streptomyces* but also other various bacteria. For example, *ArsB* and *ArsC*, both of which are type III PKSs in *Azotobacter vinelandii*, catalyze the synthesis of alkylresorcinols and alkylpyrones, respectively, which are essential for encystment as the major lipids in the cyst membrane (3).

We have recently completed the genome project for *S. griseus* and found another gene encoding a type III PKS, in addition to *RppA*. This type III PKS was named *SrsA* (*Streptomyces resorcinol synthesis*) because of its ability to synthesize alkylresorcinols in a unique manner (see below). *SrsA*, showing 31% amino acid sequence identity to *RppA*, appeared to be a member of the proteins whose synthesis was directed by an *srs* operon consisting of three genes, *srsA*, *srsB*, and *srsC* (see Fig. 1). In this paper, we report the function of the *srs* operon by elucidating the *in vivo* and *in vitro* enzymatic properties of the *Srs* products. *SrsA* was responsible for synthesis of the amphiphilic skeletons of phenolic lipids, alkylresorcinols and alkylpyrones, from acyl-CoAs of various chain lengths, as determined by *in vitro* experiments. Consistent with this idea, overexpression of *srsA* in a heterologous host, *Streptomyces lividans*, led to the accumulation of phenolic lipids. In contrast with *ArsB* and *ArsC* in *A. vinelandii*, *SrsA* used both methylmalonyl-CoA and malonyl-CoA as extender substrates, and the rank order of assembly of the extender substrates was strictly controlled. When *srsA* and *srsB* were co-expressed in *S. lividans*, alkylresorcinol methyl ethers were produced, whereas co-expression of *srsA*, *srsB*, and *srsC* led to production of hydroxylated alkylresorcinols. These observations showed that *SrsB* was a methyl transferase acting on alkylresorcinols, and *SrsC* was a hydroxylase acting on alkylresorcinol methyl ethers.

In addition to the function of the *srsABC* operon, we observed a possible role for the metabolites, alkylresorcinols and alkylpyrones. An *S. griseus* mutant deficient in the phenolic lipids synthesis was highly sensitive to penicillin G and cephalaxin, inhibitors of cell wall synthesis, which suggests that the
phenolic lipids, presumably integrated and orientated in the cytoplasmic membrane, confer rigidity of the membrane, thus permitting *S. griseus* to grow in the presence of a higher concentration of the β-lactam antibiotics. Together with the fact that a pair of genes encoding SrsA- and SrsB-like proteins is distributed widely in both Gram-positive and -negative bacteria, we speculate that the phenolic lipids play a significant, but so far unrecognized, role in the biological membranes. An attractive example is the phenolic lipids that are essential for cyst formation in *A. vinelandii* (3).

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media—** *Escherichia coli* JM109, plasmid pUC19, restriction enzymes, and other DNA-modifying enzymes used for DNA manipulation were purchased from Takara Biochemicals (Shiga, Japan). The media, growth conditions, and general recombinant DNA techniques were described by Sambrook and co-workers (4). *S. griseus* IFO13350 was obtained from the Institute of Fermentation, Osaka (IFO). *S. lividans* TK21 was obtained from D. A. Hopwood (5). For expression of *srsA*, *srsB*, and *srsC* in *Streptomyces*, pIJ6021 containing a thiostrepton-inducible *tipA* promoter (6) was used. pKUM10 (7) with its copy number of one to two/chromosome was used as a *Streptomyces* vector. For production of His-tagged SrsA in *S. lividans* TK21, pSH19 containing an ε-caprolactam-inducible *nita* promoter (8) was used. *S. lividans* and *S. griseus* were cultured in yeast extract-malt extract medium (5) and YMMD medium (0.2% yeast extract (Difco), 0.2% meat extract (Kyokuto), 0.4% Bacto peptone (Difco), 0.5% NaCl, 0.2% MgSO₄·7H₂O, 1% glucose, pH 7.2), respectively. Nucleotide sequences were determined with the Amersham Biosciences Thermo Sequenase fluorescence-labeled primer cycle sequencing kit on an automated DNA sequencer. All PCR's were conducted using the chromosomal DNA of *S. griseus* IFO13350 as the template if the template is not indicated. The absence of undesired alterations during PCR was checked by nucleotide sequencing.

**Construction of pIJ6021-srsA (pFM1)—** For construction of pFM1 containing *srsA* under the control of the *tipA* promoter in pIJ6021 (see Fig. 1), an Ndel site was introduced at the start codon of *srsA* by PCR with primer I: 5'-GGGAGATCCCATATGACCCGCATGCCGCGGT-3' (with an EcoRI site shown by underlining and the nucleotides to be changed shown by the italic letters) and primer II: 5'-GGGAGGTACGCGAGATCTCTTCGAGTCTGTGGTCTCTCCTCCT-3' (with a HindIII site shown by underlining, a BamHI site shown by the italic letters, and the start codon of *srsB* shown by the boldface letters). The 1.1-kb amplified fragment was cloned between the EcoRI and HindIII sites of pUC19, resulting in pUC19-*srsA*. The Ndel-BamHI fragment excised from pUC19-*srsA* was cloned between the Ndel and BamHI sites of pIJ6021, resulting in pFM1.

**Construction of pIJ6021-srsAB (pFM2)—** For construction of pFM2 containing *srsA* and *srsB* (see Fig. 1), the 1.6-kb fragment was amplified by PCR with primer I and primer III (5'-GGGAGGTTACTCGAGTCGAGTCTCGAGGCGAGGTGCAG-3' with a HindIII site shown by the italic letters). The amplified fragment was cloned between the EcoRI and HindIII sites of pUC19, resulting in pUC19-*srsAB*. The Ndel-HindIII fragment excised from pUC19-*srsAB* was cloned between the Ndel and HindIII sites of pIJ6021, resulting in pFM2.

**Construction of pIJ6021-srsABC (pFM3)—** For construction of pFM3 containing *srsA*, *srsB*, and *srsC* (see Fig. 1), the 1.1-kb *srsC* sequence was attached to the *srsAB* sequence in pFM2. The 1.1-kb *srsC* sequence from a BamHI site located 50 bp upstream from the stop codon of *srsB* to the nucleotide 68 bp downstream from the stop codon of *srsC* was amplified by PCR with primer IV (5'-GGGAGATCCCATATGACCCGCATGCCGCGGT-3' with a BamHI site shown by the italic letters) and primer V (5'-GGGAGGTACGCGAGATCTCTTCGAGTCTGTGGTCTCTCCTCCT-3' with a HindIII site shown by the italic letters). The BamHI-HindIII fragment was cloned between the BamHI and HindIII sites of pUC19, resulting in pUC19-*srsC*. The 1.1-kb BamHI-HindIII fragment excised from pUC19-*srsC* was cloned between the BamHI and HindIII sites of pUC19-*srsAB*, resulting in pUC19-*srsABC*. The 2.7-kb Ndel-HindIII fragment excised from pUC19-*srsABC* was cloned between the Ndel and HindIII sites of pIJ6021, resulting in pFM3.

**Construction of pKUM10-srsA—** A 0.8-kb DNA fragment from the nucleotide 480 bp upstream of the *srsA* start codon to a SalI site located 380 bp downstream of the *srsA* start codon was amplified by PCR with primer VI (5'-GGGAGATCCCATATGACCCGCATGCCGCGGT-3' with a HindIII site shown by the italic letters) and primer VII (5'-GGGAGGTACGCGAGATCTCTTCGAGTCTGTGGTCTCTCCTCCT-3' with a SalI site shown by the italic letters). The HindIII-Sall fragment was cloned between the HindIII and SalI sites of pUC19, resulting in pUC19-*srsAN*. The Sall-BamHI fragment excised from pUC19-*srsAB* was cloned between the Sall and BamHI sites of pUC19-*srsAN*, resulting in pUC19-*srsABN*. The EcoRI-BamHI fragment excised from pUC19-*srsABN* was cloned between the EcoRI and BamHI sites of pKUM10, resulting in pKUM10-*srsA*.

**Construction of pSH19-*srsA—** For adding a histidine tag to the C terminus of *srsA*, *srsA* was cloned in pET26b by introducing an Ndel and BamHI sites in the nucleotide sequences covering the start and stop codons, respectively, by PCR. PCR was performed with primer I and primer VIII (5'-GGGAGGTTACTCGAGTCGAGTCTCGAGGCGAGGTGCAG-3' with a HindIII and BamHI sites shown by underlining and the italic letters, respectively). The amplified fragment was cloned between the EcoRI and HindIII sites of pUC19, resulting in pUC19-*srsA26*. The Ndel-BamHI fragment excised from pUC19-*srsA26* was cloned between the Ndel-BamHI sites of pET26b, resulting in pET26b-*srsA*. The nucleotide sequence of *srsA* together with the histidine tag sequence in pET26b-*srsA* was amplified by PCR with primer IX (5'-GGGAGGTACGCGAGTCTCGAGGCGAGGTGCAG-3' with a PstI site shown by the italic letters; the sequence in the boldface letters is the *nita* recognition sequence, including the Shine-Dalgarno-sequence shown by underlining) (8) and primer X (5'-GGGAGATCCCATATGACCCGCATGCCGCGGT-3' with a KpnI site shown by the italic letters). The amplified fragment was cloned between the PstI and KpnI sites of pUC19, resulting in pUC19-*srsAH*. The PstI-KpnI fragment was cloned between the PstI and KpnI sites of pSH19, resulting in pSH19-*srsA*.
Phenolic Lipid Synthesis by Type III PKS in Streptomyces

S. lividans TK21 harboring pFM1, pFM2, or pFM3 was inoculated to 100 ml of yeast extract-malt extract liquid medium containing 5 μg/ml of kanamycin and grown at 30 °C. After 24 h, 5 μg/ml of thiostrepton was added to induce the tip promoter, and the culture was continued for further 48 h. A portion of the culture broth was adjusted to pH 1.0 with 6 M HCl and extracted with ethyl acetate. After evaporation to dryness, the residue was dissolved in 10 μl of dimethyl sulfoxide for reversed phase HPLC analysis. The conditions for analytical HPLC were: ODS-80T's column (4.6 × 150 mm; Tosoh, Tokyo) eluted isocratically with 95% CH3CN and 0.1% trifluoroacetic acid in water at a flow rate of 0.8 ml/min. UV absorbance was detected at 280 nm.

Large Scale Preparation and Characterization of Phenolic Lipids—The culture conditions of S. lividans TK21 harboring pFM1 or pFM2 and S. griseus harboring pFM1 were the same as those for the analytical scale, except that the culture was scaled up to 1 liter × 3. After cultivation, the mycelia obtained by centrifugation and resuspended in 50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, and 80 mM imidazole, and eluted with 50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, and 250 mM imidazole. The purified SrsA (~1.0 mg) was dialyzed twice against 2 liters of 10 mM Tris-HCl (pH 8.0). Protein concentration was measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Purity of the recombinant SrsA was checked by SDS-polyacrylamide gel electrophoresis (supplemental Fig. S1).

In Vitro SrsA Reaction—14-Methylpentadecanoyl-CoA and 14-methylhexadecanoyl-CoA were prepared according to the method of Blecher (9). The reactions, containing 100 mM Tris-HCl (pH 7.5), 29 μg of SrsA, 200 μM starter-CoA, and 100 μM extender-CoA(s), were performed in a total volume of 300 μl. The reactions were incubated at 30 °C for 3 h before being quenched by 60 μl of 6 N HCl and extracted with 300 μl of ethyl acetate. The organic layer was evaporated, and the residue dissolved in 30 μl of methanol for HPLC analysis. The conditions for analytical HPLC were as described above.

Gene Disruption of srsA in S. griseus—An in-frame deletion in the chromosomal srsA gene was introduced by replacing the region encoding from Gly-33 to Leu-302 with a Sall recognition sequence of six nucleotides (see Fig. 1). A 1.5-kb region upstream from the Pro-32 codon was amplified by using primer XI (5′-GGGAATTCCTTCGAGCTGGTGAGTTGG-3′; with an EcoRI site shown by the italic letters) and primer XII (5′-GGGCTTCGCAGCCCGCAGCTGCGTTG-3′; with the Pro-32 codon shown by the boldface letters and a Sall site shown by the italic letters). The amplified fragment was cloned between the EcoRI and Sall sites of pUC19, resulting in pUC-ΔsrsAN. A 1.5-kb region downstream from the Ala-303 codon was amplified by using primer XIII (5′-GGGCTTCGAGCCCGCAGCTGCGTTG-3′; with the Pro-32 codon shown by the boldface letters and a Sall site shown by the italic letters) and primer XIV (5′-GGGAGCTGCGGATGCCTCCCGG-3′; with a PstI site shown by the italic letters). The amplified fragment was cloned between the EcoRI and PstI sites of pUC19, resulting in pUC-ΔsrsAC. The Sall-PstI fragment excised from the pUC19-ΔsrsAC was cloned between the Sall and PstI sites of pUC19, resulting in pUC-ΔsrsAN.

Production and Purification of SrsA—S. lividans TK21 harboring pSH19-srsA was inoculated to 2 liters of yeast extract-malt extract liquid medium containing 5 μg/ml thiostrepton and grown at 30 °C. After 48 h, ε-caprolactam (final concentration, 0.1% w/v) was added to induce the niaA promoter, and the culture was continued for a further 60 h. Mycelium was harvested by centrifugation and resuspended in 50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, and 10 mM imidazole. After sonication, cell debris was removed by centrifugation. The cleared lysate was applied to nickel-nitrilotriacetic acid spin columns (Qiagen), washed five times with 50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, and 80 mM imidazole, and eluted with 50 mM NaH2PO4 (pH 8.0), 300 mM NaCl, and 250 mM imidazole. The purified SrsA (~1.0 mg) was dialyzed twice against 2 liters of 10 mM Tris-HCl (pH 8.0). Protein concentration was measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard. Purity of the recombinant SrsA was checked by SDS-polyacrylamide gel electrophoresis (supplemental Fig. S1).

Subcellular Distribution of Phenolic Lipids in S. griseus—We fractionated the culture into the extracellular, cell wall, cell...
Phenolic Lipid Synthesis by Type III PKS in Streptomyces

FIGURE 1. Genetic organization of the srs operon in *S. griseus* and schematic representation of the DNA fragments used for plasmid construction. The open triangles indicate the direction of the promoters on the vectors. The regions on pUC19-srsA-N and pUC19-srsA-C, which were used for construction of mutagenic plasmids, pUC19-ΔsrsA K, are indicated. Mutant ΔsrsA had a deletion from the 3' -end of the DNA fragment on pUC19-ΔsrsA-N to the 5' -end of the fragment on pUC19-ΔsrsA-C. The 6-kb DNA sequence covering open reading frame 1 (orf1), SrsA, SrsB, SrsC, and open reading frame 2 (orf2) has been deposited to the DDBJ data base under accession number AB425192.

membrane, and cytoplasmic fractions, according to the method of Pertwiningrum *et al.* (11). The wild-type strain *S. griseus* IFO13350 and mutant ΔsrsA were grown in 50 ml of liquid YMPD medium at 30 °C for 48 h. The cells were collected from 5 ml of culture by centrifugation at 10,000 × g. The supernatant was collected to prepare the extracellular fraction. The cells were washed with 5 ml of Tris-HCl (pH 7.5) containing 0.4 M sucrose and suspended in 5 ml of Tris-HCl (pH 7.5) containing 0.4 M sucrose and 1 mg/ml lysozyme. The mixture was incubated at 37 °C for 1 h to prepare spheroplasts. The formation of spheroplasts was checked by light microscopic observation. The spheroplasts were collected by centrifugation at 4,000 × g. The supernatant was collected to prepare the cell wall fraction. The spheroplasts were washed with 5 ml of Tris-HCl (pH 7.5) containing 0.4 M sucrose, and then 3 ml of water was added to burst the spheroplasts. The suspension was centrifuged at 100,000 × g for 1 h. The supernatant was the cytoplasmatic fraction, and the precipitate was the membrane fraction. All of the fractions were acidified with 1 M HCl and extracted with ethyl acetate. The organic layer was evaporated, and the residue was dissolved in 20 µl of methanol for HPLC analysis. The conditions for analytical HPLC were as described above.

RESULTS

Organization of the srs Operon and Homology Search of Srs Proteins—The gene organization of the 3-kb srs genes is shown in Fig. 1. The stop codon of srsA is 13 nucleotides upstream from the start codon of srsB, and the TGA stop codon of srsB is overlapped with the GTG start codon of srsC. The organization of the srs genes suggested that these three formed an operon that was transcribed from a promoter upstream of srsA. orf1, encoding a protein weakly homologous to a prenyltransferase UbiA (12), which is located 118 nucleotides upstream of srsA or orf2, encoding a putative transmembrane efflux protein, which is located 217 nucleotides downstream of srsC, that appeared not to be functionally linked to the srs operon. We did not study orf3 or orf2 further because the srs operon was sufficient for production of phenolic lipids in *S. lividans* (see below).

SrsA, consisting of 350 amino acid residues, shared 61% amino acid sequence identity to pks10 that is annotated as a functionally unknown type III PKS and is widely distributed among mycobacteria. SrsA also showed 57% identity to pks11 in *Mycobacterium tuberculosis* (supplemental Fig. S2), a type III PKS that catalyzes α-pyrene synthesis from long chain aliphatic acyl-CoA substrates (13). The similarity of SrsA to pks11 did not necessarily predict the catalytic property of SrsA, because the properties of pks11 were determined only by *in vitro* reaction and because type III PKSs are promiscuous with respect to substrate specificity and produce α-pyrones from various starter molecules (1).

A BLAST search revealed that SrsB, consisting of 177 amino acid residues, contained an isopenicysteine carboxyl methyltransferase domain of the isopenicysteine carboxyl methyltransferase family members that are unique membrane proteins involved in post-translational modification of oncogenic proteins (14). Because srsB is conserved in various bacterial species and, in most cases, consists of an operon with a type III PKS gene (supplemental Fig. S3), we assumed that SrsB was concerned with a modification of the polyketides produced by SrsA. Sequence alignment of SrsB with Ste14p (15), an isopenicysteine carboxyl methyltransferase family protein in *Saccharomyces cerevisiae*, along with a computational search for secondary structure prediction of membrane proteins, predicted that SrsB possessed two transmembrane helices (supplemental Fig. S4).

SrsC, consisting of 338 amino acid residues, contained a sequence motif found in flavoprotein hydroxylases (16) (supplemental Fig. S5), suggesting that SrsC could be a FAD-dependent hydroxylase. srsC is conserved among actinomycetes as a member of the operons including srsA and srsB in the same order (supplemental Fig. S3).

Analysis of Lipids Produced by *S. lividans* Overexpressing srs Genes—We constructed three plasmids, pFM1 carrying srsA, pFM2 carrying srsA and srsB, and pFM3 carrying srsA, srsB, and srsC and introduced them into a heterologous host, *S. lividans* TK21 (Fig. 1). The srs genes were under the strong, thioester-proton-inducible tipA promoter on the high copy number plasmid pIJ6021 for overexpression of the srs genes. We analyzed extracts from culture broth and mycelium by HPLC, on the assumption that phenolic lipids might be produced in large amounts and leaked in the culture broth in detectable amounts. Under the conditions employed, *S. lividans* harboring only the empty vector produced no detectable phenolic lipids (Fig. 2A). On the other hand, the recombinant *S. lividans* strains harboring the srs genes produced several lipids, giving multiple peaks on HPLC (Fig. 2, B–D).

For structural determination by NMR analysis of the lipids that were contained in the peaks in Fig. 2, lipids were separated by HPLC equipped with a preparative C4 column. As a result, we separated lipids in a total of 17. We determined their chemical structures (Fig. 2E) by proton and carbon NMR (supplemental text), with the aid of heteronuclear multiple quantum
correlation and heteronuclear multiple bond correlation analysis (data not shown), and LC-APCIMS and LC-APCIMS/MS analysis (supplemental Table S1). S. lividans harboring pFM1 produced alkylresorcinols (compounds 4-7) as major products and an alkylpyrone (compound 1) as a minor product. SrsA was therefore a type III PKS responsible for phenolic lipid synthesis from the substrates produced by the wild-type S. lividans strain.

On the other hand, S. lividans harboring pFM2 produced alkylresorcinol methyl ethers (compounds 8-11), suggesting that SrsB was a methyltransferase acting on alkylresorcinols. In fact, alkylpyrones 1-3 remained unmethylated, whereas alkylresorcinols 4-7 were completely methylated to the corresponding alkylresorcinol methyl ethers (compounds 8-11), which indicated the substrate preference of SrsB toward alkylresorcinols to alkylpyrones. In addition, the methoxy groups of alkylresorcinol methyl ethers were restricted to position 5 of the benzene ring (Fig. 2E). These findings implied strong substrate- and regio-specificities of SrsB as a methyltransferase.

S. lividans harboring pFM3 produced alkylquinones 15-17. In this case, we observed gradual decreases of the two peaks actually containing three compounds, 12, 13, and 14, with concomitant increases in the amounts of alkylquinones 15, 16, and 17, suggesting that the alkylquinones were derived from compounds 12-14. The structures of these compounds were deduced as 12, 13, and 14 (Fig. 2E), because the molecular masses of 12, 13, and 14 were equal to \([M_\text{w} + 2]\) Da of 15, 16, and 17, respectively. These observations led us to assume that SrsC was a hydroxylase responsible for regio-specific hydroxylation of alkylresorcinol methyl ethers, yielding alkylquinones via unstable hydroxylated intermediates (see Fig. 5A).

In Vitro SrsA Reaction—An interesting finding from the in vitro experiments with the recombinant S. lividans cells was that all the phenolic lipids produced in vivo possessed a C-methyl group on their aromatic rings. To determine whether the C-methyl group is introduced during assembly of polyketide chains by the action of SrsA, we performed in vitro reactions using recombinant SrsA with a structure of SrsA (Met-1 to Trp-350) -DPNSSSVDKLAAM-His6. The SrsA protein was used by S. lividans carrying srsA under the control the nitA promoter on pSH19, because it was difficult to prepare SrsA in a fully active form by using a pET system of E. coli in some unknown way. Recombinant SrsA thus purified from S. lividans migrated as a major protein band at a position of ~37 kDa (39,931 for the calculated molecular weight) on SDS-polyacrylamide gel electrophoresis (supplemental Fig. S1).

14-Methylpentadecanoyl-CoA (iC16) and 14-methylhexadecanoyl-CoA (iC17) were chosen as representatives for analysis of in vitro activity of the recombinant SrsA. Alkylresorcinol (compound 5), the major product of S. lividans harboring pFM1, was expected to be synthesized from iC16, methylmalonyl-CoA, and two malonyl-CoA molecules. As we expected, SrsA gave 4-methyl-5-(13'-methyltetradecyl)-benzene-1,3-diol (compound 5) as a major product and 6'-15'-dimethyl-2'-oxo-hexadecyl)-4-hydroxy-3-methylpyran-2-one (compound 1) in a very small amount from iC16, malonyl-CoA, and methylmalonyl-CoA (Fig. 3A). The rate of 1 formation was not affected by the pH change from 6.5 to 9.0, whereas the maximum rate of 5 formation was observed at pH 8.5 (data not shown). When iC16 and methylmalonyl-CoA were contained in the reaction mixture, SrsA gave a triketide pyrone, 4-hydroxy-3,5-dimethyl-6-(13'-methyltetradecyl)-pyran-2-one (compound 18), in a small amount (Fig. 3B). The structure of 18 was deduced from its [M + H]+ ion observed by LC-APCIMS analysis. In contrast, SrsA gave no products when malonyl-CoA was used as a sole extender substrate (Fig. 3C).

Similar product profiles were observed for 14-methylhexadecanoyl-CoA (aiC17)-primed reactions (Fig. 3, E and F). The major product of SrsA from aiC17, malonyl-CoA, and methylmalonyl-CoA was 4-methyl-5-(13'-methylpentadecyl)-benzene-1,3-diol.
Phenolic Lipid Synthesis by Type III PKS in Streptomyces

Involvement of srsA in Phenolic Lipid Synthesis in S. griseus—To confirm the involvement of the srs operon in biosynthesis of the phenolic lipids in vivo in the original S. griseus strain, we inactivated the chromosomal srsA gene by replacing the region encoding Gly-33 to Leu-302 of SrsA with a SalI recognition site, generating mutant ΔsrsA (Fig. 1). In relation to increased β-lactam sensitivity of mutant ΔsrsA (see below), we also analyzed the subcellular localization of the phenolic lipids by fractionating the culture into the extracellular, cell wall, cell membrane, and cytoplasmic fractions. As we expected, alkylquinones 15-17 and alkylresorcinol methyl ethers 9-11 were detected in all the fractions prepared from S. griseus IFO13350, but no phenolic lipids were detected in any fractions prepared from mutant ΔsrsA. srsA was therefore responsible for alkylresorcinol synthesis in S. griseus. The total phenolic lipids were distributed in the extracellular fraction (13%), cell membrane fraction (14%), cytoplasmic fraction (26%), and cell wall fraction (47%), when estimated by integration of the peak areas containing the respective lipids. Fig. 4A shows HPLC analysis of the cell wall fractions prepared from the wild-type and mutant strains. The amounts of alkylquinones 15-17 and alkylresorcinol methyl ethers 9-11 in all the fractions, except for the extracellular fraction, were roughly estimated to be 

A) 15 > B) 16 > C) 17 > D) 9 > E) 10 > F) 11. The amounts of alkylquinones 15-17 in the extracellular fraction were extremely small (data not shown), which suggested that the alkylquinones were localized mainly in the membrane and cell wall fractions.

Phenolic Lipids Confer Penicillin Resistance on S. griseus—We previously found that alkylresorcinols and alkylpyrones in the cysts of A. vinelandii are essential for encystment. These phenolic lipids are biosynthesized by a type III PKS, ArsB, which synthesizes 5-n-heneicosylresorcinol by condensing three molecules of malonyl-CoA onto n-behenyl-CoA (3). Because the amphiphilic property of the alkylresorcinols contributes to the formation of stable monomolecular layers in vitro (17), we assume that the phenolic lipids confer resistance to desiccation and heat on the dormant cyst of A. vinelandii. Because the phenolic lipids in S. griseus are also amphiphilic compounds, we compared growth and morphological differentiation of the wild-type strain and mutant ΔsrsA to examine a possible role for the phenolic lipids. Mutant ΔsrsA grew normally in liquid medium and on agar medium, which showed that the phenolic lipids had no detectable effects on growth or morphological development. However, we found that mutant ΔsrsA was more sensitive to β-lactam antibiotics, penicillin G, and cephalaxin, inhibitors of peptidoglycan synthesis of Gram-positive and -negative bacteria, than the wild-type strain (Fig. 4B). The wild-type S. griseus strain grew on medium containing up to 25 μg/ml of penicillin G, whereas mutant ΔsrsA did not grow on this medium. Cephalaxin also gave a similar tendency. Because both the wild-type strain and the mutant showed resistance to more than 100 μg/ml ampicillin, which is used routinely in the genetic study, we did not further examine sensitivity to this β-lactam antibiotic.

Introduction of srsA on a low amount (Fig. 3A). When aiC17 and methylmalonyl-CoA were contained in the reaction mixture, SrsA gave 4-hydroxy-3,5-dimethyl-6-(13′-methylpentadecyl)-pyran-2-one (compound 19) in a small amount (Fig. 3B).

All of these results showed that the C-methyl group on the aromatic ring was introduced through the polyketide skeleton synthesis by SrsA. SrsA was thus a novel type III PKS that catalyzed the synthesis of alkylresorcinols from a long branched chain starter substrate, one methylmalonyl-CoA molecule and two malonyl-CoA molecules.

FIGURE 3. HPLC chromatograms of products from in vitro reactions of SrsA with a starter substrate, malonyl-CoA, and methylmalonyl-CoA. The starter and extension substrates used were: 14-methylpentadecanoyl-CoA (iC15), methylmalonyl-CoA (mm), and malonyl-CoA (m) (A), iC16, and methylmalonyl-CoA (B), iC17, and malonyl-CoA (C), 14-methylhexadecanoyl-CoA (aiC16), methylmalonyl-CoA, and m (E), aiC17, and methylmalonyl-CoA (F), and aiC17, and malonyl-CoA (G). As negative controls, boiled SrsA was incubated with iC16 + methylmalonyl-CoA + malonyl-CoA (D) and with aiC17 + methylmalonyl-CoA + malonyl-CoA (H).

(compound 6), with 6-(1′,16′-dimethyl-2′-oxoheptadecyl)-4-hydroxy-3-methylpyran-2-one (compound 3) in a very small amount (Fig. 3A). When aiC17 and methylmalonyl-CoA were contained in the reaction mixture, SrsA gave 4-hydroxy-3,5-dimethyl-6-(13′-methylpentadecyl)-pyran-2-one (compound 19) in a small amount (Fig. 3B).
Phenolic Lipid Synthesis by Type III PKS in Streptomyces

DISCUSSION

We have demonstrated that the srsABC operon is responsible for phenolic lipid synthesis in S. griseus (Fig. 5A). SrsA is a type III PKS that catalyzes the synthesis of alkylresorcinols from CoA esters of branched chain fatty acids as starter substrates and both methylmalonyl-CoA and malonyl-CoA as extender substrates. SrsB acts as a post-polyketide modification enzyme to catalyze regiospecific methylation of the phenol group of alkylresorcinols, yielding alkylresorcinol methyl ethers. SrsC then catalyzes regiospecific hydroxylation of the alkylresorcinol methyl ethers, followed by nonenzymatic oxidation of the unstable hydroquinones, resulting in the formation of alkylquinones. Because the recombinant S. lividans expressing SrsA and SrsB produced alkylresorcinol methyl ethers and because only very small amounts of alkylresorcinol methyl ethers remained in S. lividans expressing SrsA, SrsB, and SrsC (Fig. 2D), we conclude that the O-methylation of the alkylresorcinols by SrsB occurs before the hydroxylation of alkylresorcinols by SrsC.

The alkylresorcinols produced by the actions of ArsA to ArsD encoded by the ars operon in A. vinelandii and by SrsA to SrsC encoded by the srs operon in S. griseus are different in chemical structure. Concerning the ArsABCD enzymes, we have recently revealed that ArsB and ArsC are type III PKSs that synthesize alkylresorcinols and alkylpyrones from the substrates produced by cooperative actions of type I fatty acid synthases ArsA and ArsD (3, 18). Because of the absence of genes encoding post-polyketide modification enzymes within the ars operon or in the A. vinelandii chromosome, the alkylresorcinols and alkylpyrones in this strain are not further modified. On the other hand, the alkylresorcinols in S. griseus are efficiently modified by the post-polyketide modification enzymes SrsB and SrsC. An additional difference is that the hydrophobic moieties of the alkylresorcinols in S. griseus contain methylmalonyl-CoA units, whereas those in A. vinelandii are derived only from straight chain fatty acids. It is known that the fatty acids of Streptomyces are biosynthesized from amino acid degradation products methylbutyryl- and isobutyryl-CoA as starter units by type II fatty acid synthase and therefore consist primarily of branched chain fatty acids (19). Because SrsA accepted CoA esters of n-fatty acids to some extent (data not shown), we suppose that the difference in the structure of starter moiety results from the in vivo availability of the substrate but not from the difference in starter substrate specificity of SrsA and ArsB.

The reaction of SrsA is different from that of ArsB in that SrsA uses one methylmalonyl-CoA and two malonyl-CoAs as extender substrates, whereas ArsB uses only malonyl-CoA. In addition, SrsA synthesizes no polyketides when malonyl-CoA is used as a sole extender substrate (Fig. 3). A unique catalytic property of SrsA is therefore that it synthesizes alkylresorcinols with an aromatic ring having a C-methyl group that is derived from methylmalonyl-CoA as an extender substrate. Although some type III PKSs can use methylmalonyl-CoA as an extender substrate (1), the alkylresorcinol synthesis by SrsA is unique because the order of condensation of methylmalonyl- and malonyl-CoA is strictly regulated in the order of malonyl-CoA, methylmalonyl-CoA, and methylmalonyl-CoA (Fig. 5B). When meth-
ylmalonyl-CoA happens to be accepted as the first extender substrate, the resultant polyketide chains are not cyclized to form resorcinols, but converted to minor products, pyrones (Fig. 5B). It may be possible that the alkylresorcinol synthesis occurs by a nucleophilic attack on the thioester by the methine carbon of intermediate A (Fig. 5B), although this route is unlikely because the acidity of a methine proton is lower than that of a methylene proton. Subsequent condensation of malonyl-CoA and methylmalonyl-CoA in this order results in synthesis of the tetraketide pyrones 1-3, whereas condensation of methylmalonyl-CoA alone gives the triketide pyrones 18 and 19. In the case of the tetraketide pyrone synthesis from intermediate B, the pyrone formation occurs as a result of a nucleophilic attack on the thioester by the oxoanion to cyclize into pyrones, instead of a nucleophilic attack by the methine carbon to yield resorcinols. This observation excludes the possibility that the alkylresorcinols are synthesized from intermediate A because of no occurrence of a nucleophilic attack by the methine carbon.

SrsA shows considerable similarity in amino acid sequence to mycobacterial type III PKSs (Pks10 and Pks11) and to SCO7671 in Streptomyces coelicolor A3(2) (supplemental Fig. S2). Pks11 synthesizes alklypyrones from long chain starter CoA and malonyl-CoA, as revealed by in vitro analysis (13). A difference between the reactions of SrsA and Pks11 is that malonyl-CoA alone is a poor substrate for SrsA. In vitro analysis of SCO7671 has shown that it accepts an acyl moiety from both hexanoyl-CoA and hexanoyl-ACP (20). The use of an acyl-ACP as a starter substrate may also be possible for the SrsA reaction, which uses thioesters of iso- and anteiso-long chain fatty acids as starter substrates, because the de novo products by bacterial type II fatty acid synthases are released as ACP esters (21) and because the CoA thioesters of long chain fatty acids are presumably absent in vivo.

The finding that the phenolic lipids confer penicillin resistance on S. griseus is interesting and important. Because the amphiphilic phenolic lipids, having an aromatic ring with a long hydrophobic alkyl chain, are presumably integrated and orientated in the membrane as for the phospholipids, they might affect the characteristics and rigidity of the membrane. Although the cyst membrane of A. vinelandii contains large amounts of the phenolic lipids, the cytoplasmic membrane of S. griseus perhaps contains them in small populations. Depending on the membranes where the phenolic lipids are localized, they could play a physiological role, for example, as the major building blocks in the dormant cyst of A. vinelandii and as minor lipids in the lipid bi-layer of the cytoplasmic membrane conferring penicillin resistance in S. griseus.

Consistent with the idea that the phenolic lipids in S. griseus play a physiological role as membrane lipids, the srs operon that is transcribed from early growth stage is not controlled by A-factor (22, 23); DNA microarray analysis showed that srsABC is transcribed at almost the same level in the A-factor-positive
and -negative backgrounds. In S. griseus, the biosynthesis of almost all secondary metabolites is under the control of AdpA (22). In fact, we have also detected the phenolic lipids in an adpA-disrupted mutant strain (data not shown), in which neither secondary metabolite formation nor morphological development occurs because of the necessity of AdpA for these processes in S. griseus (22, 24). Therefore, the phenolic lipids whose synthesis is directed by the srs operon are not merely secondary metabolites. The property of the membrane containing the phenolic lipids, as observed in S. griseus, may hold true for a wide variety of Gram-positive and -negative bacteria. Many bacteria containing an srsAB-like operon (supplemental Fig. S3) probably produce alkylresorcinols. Further study may reveal the important, but so far unrecognized, role of the phenolic lipids localized in the membranes in bacteria, fungi, and plants.

Alkylresorcinols are distributed widely in plants, fungi, and bacteria. However, there are only a few reports of phenolic lipids isolated from actinomycetes, a Streptomyces sp. (25) and Mycobacterium leprae (26). Panosilains (25), such as 5-(13-methyltetradecyl)benzene-1,3-diol, were isolated as inhibitors of a viral sialidase. We speculate that panosilains are synthesized by an SrsA-like enzyme, because they are similar in chemical structure to compounds 9-11. Whether panosilains in this Streptomyces strain play a role similar to the alkylresorcinols in S. griseus or are just secondary metabolites is unclear.

In conclusion, we have determined the role of the srs operon in S. griseus, which is responsible for phenolic lipid synthesis. SrsA uses thioesters of long chain branched fatty acids as starter substrates and condenses extender units in the order of malonyl-CoA, malonyl-CoA, and methylmalonyl-CoA. The resultant polyketide chain is cyclized into alkylresorcinols by aldol condensation. This is the first example of a type III PKS that assembles methylmalonyl-CoA and malonyl-CoA in the strictly controlled order. These intriguing catalytic properties of SrsA are worthy of future structural and mechanistic studies. Another important finding is that the phenolic lipids, probably associated with the cytoplasmic membrane in an unknown manner, affect the characteristics and properties of the cytoplasmic membrane, thus conferring resistance to -lactam antibiotics on the host.

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