Biosynthetic Investigations of Lactonamycin and Lactonamycin Z: Cloning of the Biosynthetic Gene Clusters and Discovery of an Unusual Starter Unit^{∇†}

Xiujun Zhang,¹ Lawrence B. Alemany,¹ Hans-Peter Fiedler,²
Michael Goodfellow,³ and Ronald J. Parry^{1*}

Department of Chemistry, MS60, Rice University, 6100 Main St., Houston, Texas 77005¹; Mikrobiologisches Institut, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany²; and School of Biology, University of Newcastle, Newcastle upon Tyne NE1 7RU, United Kingdom³

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The antibiotics lactonamycin and lactonamycin Z provide attractive leads for antibacterial drug development. Both antibiotics contain a novel aglycone core called lactonamycinone. To gain insight into lactonamycinone biosynthesis, cloning and precursor incorporation experiments were undertaken. The lactonamycin gene cluster was initially cloned from *Streptomyces rishiriensis*. Sequencing of ca. 61 kb of *S. rishiriensis* DNA revealed the presence of 57 open reading frames. These included genes coding for the biosynthesis of L-rhodinose, the sugar found in lactonamycin, and genes similar to those in the tetracenomycin biosynthetic gene cluster. Since lactonamycin production by *S. rishiriensis* could not be sustained, additional proof for the identity of the *S. rishiriensis* cluster was obtained by cloning the lactonamycin Z gene cluster from *Streptomyces sanglieri*. Partial sequencing of the *S. sanglieri* cluster revealed 15 genes that exhibited a very high degree of similarity to genes within the lactonamycin cluster, as well as an identical organization. Double-crossover disruption of one gene in the *S. sanglieri* cluster abolished lactonamycin Z production, and production was restored by complementation. These results confirm the identity of the genetic locus cloned from *S. sanglieri* and indicate that the highly similar locus in *S. rishiriensis* encodes lactonamycin biosynthetic genes. Precursor incorporation experiments with *S. sanglieri* revealed that lactonamycinone is biosynthesized in an unusual manner whereby glycine or a glycine derivative serves as a starter unit that is extended by nine acetate units. Analysis of the gene clusters and of the precursor incorporation data suggested a hypothetical scheme for lactonamycinone biosynthesis.

Lactonamycin (compound 1 [Fig. 1]) is an antibiotic of novel structure isolated from *Streptomyces rishiriensis* in a screen for new antibiotics active against drug-resistant bacterial strains (29, 30). Lactonamycin exhibits potent antimicrobial activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* as well as strong antitumor activity (29). Lactonamycin Z (compound 2 [Fig. 1]), a closely related antibiotic isolated from *Streptomyces sanglieri*, displays significant antitumor activity, although it exhibits weaker antibacterial activity than lactonamycin (18). Both antibiotics possess an aglycone core, lactonamycinone, whose structure is unlike that of any other antibiotic. Because of the unique structural core present in these antibiotics, they may possess an unusual mode of action that could be exploited for anticancer and antibacterial drug discovery. The presence of a carbohydrate residue in both antibiotics may also allow the creation of lactonamycinone derivatives with a new spectrum of bioactivity by changing the glycosylation pattern using combinatorial biosynthesis or glycorandomization. Both of these two techniques have been successfully employed to create libraries of glycosylated

natural product variants (7, 25). The structure of lactonamycinone suggests that these antibiotics are polyketides related to the naphthacenequinone antibiotics tetracenomycin C (TcmC) and elloramycin. The biosynthetic gene clusters for TcmC and elloramycin have both been cloned, and the functions of many of the genes in the Tcm cluster have been elucidated (11, 19, 38). Detailed investigations of TcmC biosynthesis have shown that novel oxygenase chemistry is associated with the formation of the angular *cis*-1,2-diol function found in this antibiotic (37, 54). The presence of an analogous *cis*-1,2-diol in lactonamycinone and the presence of the novel, oxygen-rich E-F ring system suggest that unusual oxygenase chemistry probably plays a role in the biosynthesis of these antibiotics. Manipulation of these post-polyketide synthase (post-PKS) tailoring enzymes may also provide access to new lactonamycinone variants (40). For these reasons, we initiated investigations to clone and characterize the lactonamycin and lactonamycin Z gene clusters.

MATERIALS AND METHODS

General methods, bacterial strains, and cloning vectors. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown and transformed as described by Sambrook et al. (41). Culture media were obtained from Becton Dickinson and Co. (Sparks, MD) unless otherwise noted. Chemical reagents were purchased from Sigma-Aldrich Co. (Milwaukee, WI). Isotopically labeled compounds were obtained from Cambridge Isotopes (Andover, MA). *Streptomyces rishiriensis* MJ773-88K4 was obtained from Tomio Takeuchi at the Institute of Microbial Chemistry, Tokyo, Japan, while *S. sanglieri*

* Corresponding author. Mailing address: Department of Chemistry, MS60, Rice University, 6100 Main St., Houston, TX 77005. Phone: (713) 348-2446. Fax: (713) 348-5155. E-mail: parry@rice.edu.

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FIG. 1. Structures of lactonamycin (compound 1) and lactonamycin Z (compound 2).

AK 623 was obtained from the culture collection at the University of Newcastle. *S. sanglieri* AK 623 was cultured at 27°C in a medium containing 1% starch, 1% glucose, 1% corn steep powder, 0.5% casein-peptone, 0.2% yeast extract, 0.1% NaCl, pH 7.0, in tap water. *S. rishiriensis* was cultured under reported conditions

(29) and under the conditions used for *S. sanglieri* AK 623. *S. rishiriensis* and *S. sanglieri* were maintained on ISP 2 (4 g/liter yeast extract, 10 g/liter malt extract, 4 g/liter dextrose, 20 g/liter agar) and ISP 3 agar (20 g/liter oatmeal extract, 1 ml/liter trace elements solution [in 100 ml H₂O, 0.1 g FeSO₄ · 7H₂O, 0.1 g MnCl₂ · 4H₂O, 0.1 g ZnSO₄ · 7H₂O]), respectively.

DNA manipulations and sequence analysis. Plasmid and cosmid DNA were purified with a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). PCR products were separated on agarose gels and purified from the gels using a GeneClean Spin kit (MP Biomedicals, Solon, OH). Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures under conditions recommended by the manufacturers. Automated DNA sequencing was performed using universal and synthetic oligonucleotide primers. Cosmids were sequenced by release of the inserts with XbaI and SpeI followed by further digestion with BamHI or PstI to generate a pool of fragments that were then cloned into pBluescript II SK(+) or pSP72 plasmid vectors. The clones were then sequenced with universal primers followed by primer walking with designed primers. DNA gaps between subclones were filled in by direct sequencing of the parent cosmid. Sequences were determined by complete sequencing of both DNA strands with multiple sequencing of some regions.

PCR amplification of an NDP-hexose 2,3-dehydratase from *S. rishiriensis*. The presence of an L-rhodinose moiety in lactonamycin suggested that a nucleoside diphosphate (NDP)-hexose 2,3-dehydratase gene should reside within the lactonamycin biosynthetic gene cluster. Accordingly, several sets of degenerate oligonucleotide primers were designed to amplify an internal region of an NDP-hexose 2,3-dehydratase gene from the lactonamycin producer *S. rishiriensis* MJ 773-88K4. The sequences of these primers were derived from conserved domains of NDP-hexose 2,3-dehydratase genes found in antibiotic biosynthetic gene clusters of *Streptomyces violaceoruber* Tu22 (*gra* ORF27) (13), *Streptomyces peucetius* (*dnmT*) (43), *Saccharopolyspora erythraea* (*eryBVI*) (14, 49), and *Streptomyces nogalater* (*snogH*) (53).

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s) ^a	Reference or source
Bacterial strains		
<i>Streptomyces rishiriensis</i> MJ773-88K4	Producer of lactonamycin	29
<i>Streptomyces sanglieri</i> AK 623	Producer of lactonamycin Z	18
<i>S. sanglieri</i> AK 623 Δ lcz36	<i>S. sanglieri</i> AK 623 with nt 40 to 1281 of <i>lcz36</i> replaced by pIJ773 <i>aac(3)IV-oriT</i> cassette; Ap ^r	This work
<i>Escherichia coli</i> DH10B	General cloning host	Invitrogen
<i>E. coli</i> 12567	<i>dam-13::Tn9 dcm-6 hsdM</i>	27
<i>E. coli</i> BW25113	K-12 derivative; Δ araBAD Δ rhaBAD	10
Plasmids		
pBluescript II SK(+)	<i>E. coli</i> cloning vector; Am ^r	Stratagene
pSP72	<i>E. coli</i> cloning vector; Am ^r	Promega
pGEM-T Easy	PCR cloning vector; Am ^r	Promega
pOJ446	<i>E. coli-Streptomyces</i> shuttle cosmid for conjugal transfer; Ap ^r	5
pIJ773	pBluescript II SK(+) containing an <i>aac(3)IV-oriT</i> cassette	16
pIJ790	λ Red (<i>gam bet exo</i>) <i>cat araC rep101</i> ^{ts} Cm ^r	16
pIJ702	pIJ101 derivative; <i>rep101</i> Ts ^r	24
pUZ8002	<i>tra neo</i> RP4	36
SuperCos1	<i>E. coli</i> cosmid cloning vector; Carb ^r Kan ^r	Stratagene
pGEM-T Easy DH35	495-bp PCR internal <i>lct45</i> fragment cloned into pGEM-T Easy	This work
pBS-VII-41F	pGEM-3zf containing <i>ermEp</i> * promoter as 0.45-kb EcoRI/SacI fragment	B. Shen, University of Wisconsin
pSET152	<i>E. coli-Streptomyces</i> Shuttle plasmid; <i>oriT int^{φC31}</i> Am ^r	5
pXZ152	pSET152 derivative with <i>tsr</i> gene; Ap ^r Ts ^r	This work
pOE152	pXZ152 derivative containing <i>ermEp</i> * promoter; Ap ^r Ts ^r	This work
pOElcz36	pOE152 with <i>lct36</i> cloned downstream of the <i>ermE</i> * promoter; Ap ^r Ts ^r	This work
pKolcz36	Cosmid pCAS2 with nt 40 to 1281 of <i>lcz36</i> replaced by pIJ773 <i>aac(3)IV-oriT</i> cassette	This work
pCAK7	pOJ446 cosmid clone of <i>S. sanglieri</i> AK 623, hybridizing to <i>lac36</i>	This work
pCAS2	SuperCos1 cosmid clone of <i>S. sanglieri</i> AK 623, hybridizing to <i>lct36</i>	This work
pCSR5	pOJ446 cosmid clone of <i>S. rishiriensis</i> MJ773-88K4 hybridizing to the pGEM-T Easy DH35 <i>lct36</i> insert	This work
pCSR8	pOJ446 cosmid clone of <i>S. rishiriensis</i> MJ773-88K4 hybridizing to the pGEM-T Easy DH35 <i>lct36</i> insert	This work
pCSR13	pOJ446 cosmid clone of <i>S. rishiriensis</i> MJ773-88K4 hybridizing to <i>lct10</i>	This work

^a Drug resistance markers used are ampicillin (Am), apramycin (Ap), carbacillin (Carb), chloramphenicol (Cm), kanamycin (Kan), and thiostrepton (Ts).

One set of degenerate primers led to the successful amplification of a 495-bp NDP-hexose 2,3-dehydratase gene fragment. The degenerate primers were N-terminal primer DHF (5'-GGSCGTTCTCTCTSGAG-3') and C-terminal primer DHR (5'-ACSGTSCSGHGTCCATGTT-3', where S is C or G).

Cosmid library construction and screening. *S. rishiriensis* and *S. sanglieri* were grown in yeast extract-malt extract (YEME) medium (24), and genomic DNA was extracted by standard methods (24). Cosmid libraries of *S. rishiriensis* and *S. sanglieri* DNA in cosmid vector pOJ446 (5) were constructed in a manner previously described (57). In a similar way, a cosmid library of *S. sanglieri* DNA in cosmid vector SuperCos1 was also constructed. Screening of the cosmid libraries by Southern hybridizations was carried out as previously described (57). The *S. rishiriensis* pOJ446 cosmid library was screened with the 495-bp NDP-hexose 2,3-dehydratase fragment amplified from *S. rishiriensis* by PCR, while the two *S. sanglieri* cosmid libraries were screened with a 1,320-bp PCR product obtained by PCR amplification of the *S. rishiriensis* *lct36* gene.

Fermentation of *S. rishiriensis* MJ773-88K4. Fermentation of *S. rishiriensis* MJ773-88K4 was carried out according to published methods (29) and also under the conditions employed for *S. sanglieri* AK 623 (see below). The levels of lactonamycin production were low in both media, and reproducible antibiotic production was difficult to maintain. Analytical high-performance liquid chromatography (HPLC) detection of lactonamycin was carried out on a 4.6- by 250-mm YMC-Pack ODA-A column as described below for lactonamycin Z. The retention time for lactonamycin in this system was ca. 27 min. Small amounts of lactonamycin could be isolated from the fermentation broth by preparative HPLC using a 10- by 250-mm YMC-Pack ODS-A column and the conditions described for preparative HPLC of lactonamycin Z (see below).

Fermentation of *S. sanglieri* and isolation of lactonamycin Z. Fermentation of *S. sanglieri* AK 623 was carried out at 27°C and 120 rpm in the medium described above. The medium (100 ml) was inoculated from a spore suspension and grown at 27°C for 48 h. Fresh medium (five 200-ml portions) was then inoculated with five 10-ml samples of the seed culture, and fermentation was allowed to proceed for 96 h. The pH of the medium was readjusted to 4.5, and the mixture was centrifuged to separate the fermentation broth from the mycelium. The supernatant was extracted three times with ethyl acetate, while the mycelium was suspended in excess 50% aqueous acetone and stirred for 1 hour. The aqueous acetone extract was filtered, and the acetone was removed in vacuo. The aqueous residue was extracted three times with ethyl acetate, and the resulting extracts were combined with the ethyl acetate extracts of the fermentation broth. The total ethyl acetate extract was dried over anhydrous sodium sulfate and filtered, and the ethyl acetate was removed in vacuo. The residue was dissolved in chloroform and filtered to remove insoluble material. The filtered chloroform extract was taken to dryness in vacuo, and lactonamycin Z was isolated from the residue by preparative HPLC as described below. The estimated titer of lactonamycin Z in the fermentation broth is ca. 5 mg per liter.

HPLC analysis of lactonamycin Z. HPLC analysis was carried out with a Varian ProStar 325 UV-visible detector set at 300 nm and a YMC-Pack ODS-A column (4.6 by 250 mm). Elution was carried out by running a linear gradient over 30 min from 40:60 methanol-water containing 0.5% formic acid to 80:20 methanol-water containing 0.5% formic acid with a flow rate of 1 ml/min. Under these conditions, lactonamycin Z exhibited a retention time of ca. 21 min. For preparative applications, the same gradient was applied to a YMC-Pack ODS-A column (10 by 250 mm) with a flow rate of 3 ml/min. Approximately 1 to 2 mg of purified lactonamycin Z could be obtained from 1 liter of fermentation broth.

Preparation of lactonamycinone from lactonamycin Z. Lactonamycinone was prepared from lactonamycin Z by a modification of the procedure used to convert lactonamycin into lactonamycinone (30). Lactonamycin Z (3.5 mg) was dissolved in 0.5 ml of spectral grade tetrahydrofuran, and 0.2 ml of 1 N HCl was added. The resulting solution was stirred at room temperature and monitored periodically by HPLC. After 67 h, the tetrahydrofuran was removed in vacuo at room temperature, and the remaining aqueous solution was diluted with 1 ml of water and extracted three times with chloroform. The combined chloroform extracts were dried, and the solvent was removed in vacuo to yield ca. 1.0 mg of lactonamycinone, which exhibited an HPLC retention time of 19 min under the same conditions used for analytical HPLC analysis of lactonamycin and lactonamycin Z. Nuclear magnetic resonance (NMR) analysis confirmed the assigned structure: ¹H NMR (500 MHz, CDCl₃, δ values), 2.99 (1H, d, J = 17.0 Hz, H-3), 3.09 (1H, d, J = 17.0 Hz, H-3), 3.20 (3H, s, OCH₃), 3.29 (3H, s, NCH₃), 4.10 (1H, d, J = 9.6 Hz, H-5), 4.91 (1H, d, J = 9.6 Hz, H-5), 4.98 (2H, s, H-12), 7.31 (1H, s, H-8), 8.07 (1H, s, H-7), 9.36 (1H, s, very broad, C-9 OH), 13.70 (1H, s, broad, C-13 OH); ¹³C NMR (125 MHz, CDCl₃), 29.2 (C-16, NCH₃), 37.7 (C-3), 52.7 (C-15, OCH₃), 55.0 (C-12), 73.8 (C-5), 82.6 (C-5a), 90.6 (C-14a), 109.4 (C-13a), 112.4 (C-3a), 112.9 (C-8), 116.6 (C-12b), 121.6 (C-7), 129.8 (C-6a), 141.8 (C-7a), 142.7 (C-12a), 157.4 (C-9), 164.0 (C-13), 168.9 (C-10), 170.6

(C-2), 189.5 (C-6), 192.2 (C-14). NMR spectral data for lactonamycinone have not been previously reported.

Inactivation of lactonamycin Z production by double-crossover disruption of the glycosyltransferase gene (*lct36*). Double-crossover disruption of the lactonamycin Z glycosyltransferase gene (*lct36*) was carried out by using the lambda Red-mediated recombination methodology (15, 16). The PCR product required for disruption of the *lct36* gene was obtained by amplification of the 1,383-bp *aac(3)/IV-oriT* sequence using pIJ773 as a template with primer pairs containing 39-nucleotide (nt) extensions derived from the 5' and 3' ends of the *lct36* gene. The forward and reverse primers were ZGLTF (5'-ATGTGTTTCGTCGCGGAAAGGATTGGA GAGCATGGATGATTCCGGGATCCGTCACC-3') and ZGLTR (5'-GGGAC CGGCCCGCCGACGACAGATGCCTGACGATTTGTATGTAGGCTGGAGC TGCTTC-3', respectively, where the underlined portions show the 39-nt extensions). Electrocompetent cells of *E. coli* BW25113 (10) containing pIJ790 (Cm^r) and the SuperCos1-derived cosmid pCAS2 (Carb^r Kan^r) containing *lct36* were prepared using the method of Gust et al. (15). The electrocompetent cells were transformed with purified PCR product using a Bio-Rad Gene Pulser II set at 200 Ω, 25 μF, and 2.5 kV. The shocked cells were incubated in 1 ml of LB broth at 37 °C for 1 h and then selected on LB agar containing ampicillin (100 μg/ml), kanamycin (50 μg/ml), and apramycin (50 μg/ml). Transformants carrying the desired cosmid, pKOlc36, were identified by restriction digestion of the isolated cosmids. Plasmid pKOlc36 was then introduced into the nonmethylating *E. coli* ET12567 (27) containing the RP4 derivative pUZ8002 (36) and transferred into *Streptomyces sanglieri* AK 623 by intergeneric conjugation. For intergeneric conjugation, spores of *Streptomyces sanglieri* AK 623 were heat shocked at 50°C for 10 min, mixed with the *E. coli* cells prepared according to a standard protocol (24), and plated out on AS1 medium (1). After incubation at 30°C for 20 h, the plates were then overlaid with 3 ml of 0.7% soft tryptic soy agar containing 0.5 mg nalidixic acid and 1.25 mg apramycin. Incubation was then continued at 30°C for 5 to 7 days. Exconjugants were selected, and their antibiotic resistance was evaluated. Cosmid DNA was isolated from Kan^r Ap^r colonies, and the presence of the expected double-crossover disruption was verified by PCR amplification of the disrupted region using the following primer set: Flc36 (5'-GGTCTTGTATCGGGAAAG-3') and Rlc36 (5'-CCAGTACGGCAGTATG CG-3'). Sequencing of the PCR product (data not shown) and restriction digests of the amplified fragment (Fig. 2) confirmed the presence of the predicted disruption. In addition, genomic DNA was extracted from the Δ*lct36* disruptant, digested with BglII, and analyzed by Southern hybridization using a 916-bp probe amplified from the *lct36-lct37* region of wild-type *S. sanglieri* using the following primer set: Lc36F (5'-CGGTACCTTCGCGTCCC-3') and Lc36R (5'-CTCCAGTGTGCGGGCG AAG-3'). The hybridization pattern exhibited by the Δ*lct36* disruptant was completely consistent with the genetic alteration expected from the double-crossover disruption (Fig. 3). Fermentation of the Δ*lct36* disruptant and analysis of the combined organic extracts of the mycelium and fermentation broth by HPLC showed that lactonamycin Z production had been abolished. A small peak (compound X) that exhibited approximately the same retention time as the aglycone lactonamycinone was also present in the extracts, but mass spectrometry analysis indicated that compound X is not lactonamycinone (Fig. 4).

Construction of plasmid pOElc36. To complement *S. sanglieri* AK 623 Δ*lct36*, a derivative of the integrating plasmid pSET152 (5) was constructed. First, the thiostrepton resistance gene (*tsr*) from plasmid pIJ702 was amplified by PCR using the following forward and reverse primers: Tsr-XbaIF (AAAAATCTAG ATCACTGACGAATCGAGGTGCGAGGAAC) and Tsr-NheIR (AAAAAGCT AGCAGGCGAATACTTCATATGCGGGGATC, where the underlined bases correspond to XbaI and NheI sites, respectively). The resulting PCR fragment was digested with XbaI and NheI and then cloned into the NheI site of pSET152. A construct with the *tsr* gene in the same orientation as *aac(3)/IV* of pSET152 was then identified by restriction digestion and designated pXZ152. The 0.45-kb *ermEp** promoter cassette of plasmid pBS-VII-41F was PCR amplified using the following forward and reverse primers: ErmMfeI (5'-AAAAACAATTGAGCC CGACCCGAGCACGC-3') and ErmEcoRI (5'-AAAAAGAAATTCATGGG GTCGACCCACCGACGAC-3', where the underlined nucleotides correspond to MfeI and EcoRI sites, respectively).

The PCR product was digested with MfeI and EcoRI and then cloned into the EcoRI site of pXZ152. A construct with *ermEp** promoter in the same orientation as the *lacZα* gene of pSET152 was identified by restriction digestion and designated pOE152 (data and map not shown). To complement the Δ*lct36* mutant, the PCR primers 5'-AAAAAGAAATTCATGAGAGTTCTGTTGC T-3' and 5'-AAAAATCTAGATCAGGACCGGTGCGGGC-3' were used to amplify the 1,284-bp *lct36* gene from *S. sanglieri* (EcoRI and XbaI sites of primers underlined). The purified PCR product was digested with EcoRI and XbaI and then cloned into the EcoRI-XbaI sites of pOE152 downstream of the *ermEp** promoter, therefore generating the expression plasmid pOElc36.

FIG. 2. Verification of *S. sanglieri* Δ *lcz36* mutant by restriction digestion of PCR products from wild-type (WT) and mutant strains. B, BglII; X, XhoI.

Complementation of the *S. sanglieri* AK 623 Δ *lcz36* mutant. Plasmid pOEIcz36 was introduced into *E. coli* ET12567/pUZ8002 and intergeneric conjugation was carried out with the Δ *lcz36* mutant in the manner described above. Exconjugants were selected using thiostrepton at 50 μ g/ml. The integration of pOEIcz36 into the chromosome of mutant Δ *lcz36* was verified by PCR (data not shown). Fermentation of *S. sanglieri* Δ *lcz36* (pOEIcz36) was carried out in the usual manner, and the combined organic extracts of the mycelium and fermentation broth were analyzed for the presence of lactonamycin Z by HPLC. The analysis showed that lactonamycin Z production had been restored (Fig. 4). This was confirmed by isolation and NMR characterization of lactonamycin Z from the complemented strain (data not shown). The level of lactonamycin Z production by the complemented strain was estimated to be ca. one-third that of the wild-type strain.

Administration of isotopically labeled precursors to *S. sanglieri* AK 623. Sterile *S. sanglieri* AK 623 culture medium (eight 200-ml portions) was inoculated with eight 10-ml samples of a 48-h culture of *S. sanglieri* grown under standard conditions. After incubation at 27°C, 120 rpm, for 48 h, a filter-sterilized aqueous solution containing 1.0 g of the labeled precursor was distributed equally to each of the eight shaking flasks, and the fermentation was allowed to continue for an additional 48 h. At the end of this time, lactonamycin Z was isolated from the fermentation broth by preparative HPLC as described above. All precursors were 99% enriched with ^{13}C . The [2- ^{13}C , ^{15}N]glycine was 98% enriched with ^{15}N .

Nucleotide sequence accession numbers. The nucleotide sequences were deposited in the GenBank database and given accession numbers EU147298 and EU147299.

RESULTS

Precursor incorporation experiments with *S. sanglieri*. The structures of lactonamycin and lactonamycin Z suggest that the aglycone portion of these antibiotics, lactonamycinone, is biosynthetically related to the decaketide TcmC. This hypothesis was evaluated by administration of sodium [1- ^{13}C]-, [2- ^{13}C]-, and [1,2- $^{13}\text{C}_2$]acetate to the *S. sanglieri* fermentation. The results of these experiments are summarized in Table 2. Several conclusions can be drawn from these data. First, the labeling patterns produced by each of these precursors are consistent with the head-to-tail incorporation of nine acetate units into lactonamycinone. Second, the data indicate that the ^{13}C chemical shift assignments reported for C-3 and C-2' of lactonamycin Z (18) need to be reversed. This conclusion follows from the labeling patterns observed with [2- ^{13}C]- and [1,2- $^{13}\text{C}_2$]acetate and is consistent with the ^{13}C chemical shift assigned to C-3 of lactonamycin (30) (see supplemental material for more detail). Third, the labeling pattern is consistent with a mechanism for formation of the E/F ring system that involves

FIG. 3. Southern hybridization analysis of *S. sanglieri* Δ *lcz36* mutant and wild-type *S. sanglieri* (WT) using a 916-bp probe derived from *lcz36* and *lcz37*. In the Δ *lcz36* mutant, *lcz36* is replaced by the FLP recombination target (FRT)-*aac(3)IV*-*oriT*-FRT cassette from pIJ773. Bg, BglII.

FIG. 4. HPLC analysis of fermentation broth from wild-type *S. sanglieri* (WT), the $\Delta lcz36$ mutant, and the complemented $\Delta lcz36$ mutant using HPLC conditions described in Materials and Methods. Z, lactonamycin Z; X, unknown.

oxidative cleavage of ring D of a naphthacenequinone intermediate similar to TcmD3 (Fig. 5). Last, the labeling patterns show that C-12 and C-12a are not derived from acetate, since none of the forms of labeled acetate produced enrichment in these carbon atoms. The absence of labeling on C-12 and C-12a suggested that an alternative starter unit is employed for

polyketide assembly. A likely candidate for a starter unit appeared to be glycine. This possibility was examined by administration of $[1,2-^{13}\text{C}_2]$ -, $[1-^{13}\text{C}]$ -, and $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine to *S. sanglieri* AK 623. The results of these experiments (Table 2) demonstrate that C-12a and C-12 of lactonamycinone are derived from C-1 and C-2 of glycine, respectively, and that the nitrogen atom in the δ -lactam ring of lactonamycinone is derived from the nitrogen atom of glycine. In addition to labeling some of the carbon atoms of the δ -lactam ring, both $[1,2-^{13}\text{C}_2]$ - and $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine led to efficient labeling of the O-methyl and N-methyl groups of the aglycone (C-15 and C-16). However, no labeling of the O- and N-methyl groups was observed when $[1-^{13}\text{C}]$ glycine was administered to the fermentation. Labeling of the O-methyl and N-methyl groups by $[1,2-^{13}\text{C}_2]$ - and $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine can be explained if C-2 of glycine is entering the C_1 pool via metabolism of the labeled amino acid by the glycine cleavage system. The glycine cleavage system is a multienzyme complex composed of four proteins that catalyzes the oxidative decarboxylation of glycine to carbon dioxide, ammonia, and a C_1 unit that is incorporated into 5,10-methylenetetrahydrofolate (34, 35). A hypothetical biosynthetic pathway for lactonamycinone based upon the results of the precursor incorporation experiments is shown in Fig. 5. In this pathway, the postulated formation of the 1,2-*cis* diol moiety of lactonamycinone by the formal *cis* ring opening of an epoxide is based upon an analogous process in TcmC biosynthesis (37), while the timing for formation of the δ -lactam ring of lactonamycinone is arbitrarily shown to occur at a late stage in the pathway.

Cloning of the lactonamycin gene cluster from *S. rishiriensis*. Initial efforts to clone the lactonamycin gene cluster were focused upon the lactonamycin producer *S. rishiriensis*. The presence of an L-rhodinose moiety in the antibiotic suggested that cloning could be accomplished by using deoxyhexose biosynthetic genes as probes. Using the conserved domains of NDP-hexose 2,3-dehydratases associated with several antibiotic biosynthetic pathways in *Streptomyces* species, degenerate primers were designed to amplify an NDP-hexose 2,3-dehydratase gene from the genomic DNA of *S. rishiriensis*. One set of primers led to the amplification of a 495-bp PCR product, which was ver-

TABLE 2. Incorporation of labeled precursors into lactonamycin Z

Precursor	Labeling pattern	One-bond coupling(s) (Hz)
Sodium $[1-^{13}\text{C}]$ acetate	C-2, C-3a, C-5a, C-6a, C-7a, C-9, C-10, C-13, C-14	None observed
Sodium $[2-^{13}\text{C}]$ acetate	C-3, C-5, C-6, C-7, C-8, C-9a, C-12b, C-13a, C-14a	None observed
Sodium $[1,2-^{13}\text{C}_2]$ acetate	C-2, C-3, C-3a, C-5, C-5a, C-6, C-6a, C-7, C-7a, C-8, C-9, C-9a, C-10, C-12b, C-13, C-13a, C-14, C-14a	C-2, C-3 = 52.7 C-5, C-5a = 40.8 C-6, C-6a = 55.3 C-7, C-7a = 55.2 C-8, C-9 = 73.0 C-9a, C-10 = 64.1 C-12b, C-13 = 67.3 C-13a, C-14 = 59.8 C-14a, C-3a = 37.1
$[1,2-^{13}\text{C}_2]$ glycine	C-12, C-12a, C-15, C-16	C-12, C-12a = 41.6
$[1-^{13}\text{C}]$ glycine	C-12a	None observed
$[2-^{13}\text{C}, ^{15}\text{N}]$ glycine	C-12, C-15, C-16, N-11	C-12, ^{15}N = 11.3

FIG. 6. Genetic organization of the *lct* gene cluster from *S. rishiriensis* and the partial *lcz* gene cluster from *S. sanglieri*.

is conceivable that Lct26 plays a role in the loading of glycine or a glycine derivative onto Lct31 (see Discussion). Both ACPs display the conserved LG(x)DS motif containing the serine residue that is the attachment site for a 4'-phosphopantetheine prosthetic group (21). The *lct8* gene appears to encode a 4'-phosphopantetheinyltransferase. The function of this enzyme may be to convert Lct24 and Lct26 from the apo form to the holo form.

Three putative polyketide cyclases, Lct27, Lct29, and Lct30, reside within the *lct* cluster. Lct27 exhibits 59% identity to FdmI, a cyclase from the fredericamycin gene cluster (55). Lct27 also shows high similarity to the N-terminal cyclase domain of the bifunctional protein TcmN from the Tcm cluster (50). Lct27 may catalyze cyclizations leading to the formation of an anthrone intermediate resembling TcmF2 (Fig. 5). Lct29 exhibits 43% identity to the cyclase TcmI (46), suggesting that Lct29 probably catalyzes a cyclization leading to an intermediate similar to TcmF1. Lct30 displays 49% identity to TcmJ. TcmJ has been shown to increase the production of TcmF2 in vitro, although the mode of action of TcmJ is unclear (2). An unusual feature of the *lct* cluster is the presence of the Lct34 thioesterase. Thioesterases are commonly found in type I PKS gene clusters and nonribosomal peptide gene clusters, but they are only rarely associated with type II PKS genes. A thioesterase gene has been reported to occur in a type II PKS gene cluster that resides on the linear pSLA2-L plasmid of *Streptomyces rochei* (32).

(ii) Genes encoding tailoring enzymes or proteins of unknown function. The lactonamycin cluster encodes a relatively large number of oxygenases and oxidoreductases. Potential functions can be assigned to only two of the oxygenases. Lct41 displays 46% identity to TcmG and 46% identity to ElmG. The latter enzymes have been shown to catalyze the triple hydroxylation of TcmA2 to TcmC, a reaction leading to formation of a *cis*-1,2-diol function (37, 47, 54). Lct41 may be involved in the formation of the similar *cis*-1,2-diol moiety found in lactonamycin. Lct33 and Lct42 exhibit strong similarities to TcmH, a monooxygenase that catalyzes the oxidation of the naphtha-

cenone TcmF1 to the naphthacenequinone TcmD3 (45). Structure determination of the related enzyme ActVA-ORF6 led to the characterization of an "antibiotic biosynthesis monooxygenase" (ABM) domain found in this family of proteins and the identification of three active site residues (Asn, Trp, and Arg) (42). Both Lct33 and Lct42 exhibit many of the conserved residues displayed by the ABM domain, but only Lct33 contains all three active site residues. This suggests that Lct33 plays a role in lactonamycin biosynthesis similar to that of TcmH in TcmC biosynthesis, while the function of Lct42 is unclear. An additional oxidative enzyme is encoded by *lct10*, whose translation product exhibits strong similarities to cytochrome P450 enzymes. Since *lct10* is situated between genes that appear to be involved in the biosynthesis and binding of a γ -butyrolactone autoregulator, it is likely that *lct10* plays a role in the biosynthesis of a γ -butyrolactone. The *lct* cluster contains one glycosyltransferase, Lct36. Lct36 is presumed to catalyze the attachment of L-rhodinose to lactonamycinone.

Two genes encoding methyltransferases are present in the *lct* cluster. Lct25 aligns with the C-terminal methyltransferase domain of the bifunctional protein TcmN. TcmN methylates the C-11 phenolic hydroxyl group of TcmD3 (44). It is possible that Lct25 methylates the C-13 hydroxyl group of a TcmD3 analog on the pathway to lactonamycin. The second methyltransferase is Lct35. This protein shows 50% identity to TcmO, an enzyme that methylates the C-3 phenolic hydroxyl of TcmB3 (50). Since the corresponding C-3 hydroxyl group of lactonamycin is unmethylated, it is possible that Lct35 is required for formation of the N-methyl group of lactonamycin.

Eight additional ORFs within the *lct* cluster encode proteins whose functions cannot be predicted from sequence analysis. The translation product of the *lct16* gene exhibits strong similarity to FAD-dependent monooxygenases that catalyze the hydroxylation of aromatic rings, while *lct40* and *lct43* encode oxidoreductases. All three of these genes might play a role in the formation of the lactonamycin E/F ring system. The protein encoded by *lct28* shows high similarity to acyl coenzyme A (acyl-CoA) synthetases and carboxylic acid adenylation enzymes. Because of the specific

TABLE 3. Organization and analysis of lactonamycin gene cluster from *S. rishiriensis*

Gene designation	Size (aa) ^a	Protein homolog(s) [GenBank accession number(s)], % similarity/% identity	Possible function(s)
<i>orf1</i>	288	SCC61A.21c (CAB892266), 88/79	Tyrosinase
<i>orf2</i>	98	SteO22.21c (CAI59990), 86/78	Putative small membrane protein
<i>orf3</i>	92	Dace_2175 (EAT15876), 58/32	Acyl carrier protein
<i>orf4</i>	360	RHA1_ro02212 (ABG94019), 51/38	Phosphatase, acyltransferase
<i>orf5</i>	191	EAV71565 (CcelDRAFT_0530), 56/35	N-Acetyltransferase
<i>orf6</i>	409	RPD_3346 (ABE40570), 55/41	Hypothetical protein, methyltransferase?
<i>orf7</i>	126	CYA_0574 (ABC98790), 61/47	S-Adenosylmethionine decarboxylase
<i>lct8</i>	298	AAP09275 (BC_2311) 48/34	4'-Phosphopantetheinyltransferase
<i>lct9</i>	314	Orf85 (BAC76543), 82/78	AfsA homolog, β -ketoacyltransferase, γ -butyrolactone biosynthesis
<i>lct10</i>	442	Orf84 (BAC76542), 75/62	Cytochrome P450, γ -butyrolactone biosynthesis?
<i>lct11</i>	254	Orf83 (BAC76541), 75/66	Phosphatase, γ -butyrolactone biosynthesis
<i>lct12</i>	249	SngB (AAX97701), 72/61	Ketoacyl acyl carrier protein/CoA reductase, γ -butyrolactone biosynthesis
<i>lct13</i>	222	TarA (AAF06961), 82/72; TylP (AAD40801), 78/68	γ -Butyrolactone binding protein
<i>lct14</i>	160	TylQ (AAD40803), 63/50	Transcriptional regulator
<i>lct15</i>	276	Orf75 (NP_851497), 79/69; TylS (AAD40804), 75/65	<i>Streptomyces</i> antibiotic regulatory protein (SARP)
<i>lct16</i>	500	SAV3380 (NP_824557), 64/52	FAD-dependent monooxygenase
<i>lct17</i>	327	Orf21 (BAC79034), 73/66	Carbohydrate kinase (glucose kinase)
<i>lct18</i>	1,133	Orf49 (BAC76507), 92/87	5-Methyltetrahydrofolate:homocysteine S-methyltransferase
<i>lct19</i>	285	Orf (+9) (AAN85549), 87/81	5,10-Methylenetetrahydrofolate reductase
<i>lct20</i>	469	MtmH (CAK50773), 97/93	Adenosylhomocysteinase
<i>lct21</i>	220	Orf71 (BAC76569), 64/53	SARP
<i>lct22</i>	509	SAV3632 (BAC71344), 53/42	AfsR-like regulatory protein
<i>lct23</i>	563	SnorA (CAA12016), 51/38	AfsR-like regulatory protein
<i>lct24</i>	92	Sim4 (AAL15582), 69/52	Acyl carrier protein
<i>lct25</i>	349	TcmN (P16559), 66/50; FdmN (AAQ08925), 58/40	O-Methyltransferase; aligns with the C-terminal region of TcmN
<i>lct26</i>	83	RPC_2301 (ABD87855), 67/47	Acyl carrier protein
<i>lct27</i>	152	FdmI (AAQ08919), 73/59; TcmN (P16559), 68/53	Polyketide cyclase; aligns with the N-terminal region of TcmN
<i>lct28</i>	510	SAV1250 (BAC68960), 63/52	Carboxylic acid adenylation
<i>lct29</i>	109	ElmI (AAF73052), 63/42; TcmI (P39890), 63/43	Polyketide cyclase
<i>lct30</i>	138	TcmJ (P16558), 70/49; ElmJ (AAF73053), 65/48	Polyketide cyclase
<i>lct31</i>	421	TcmK (P16538), 80/69	KS $_{\alpha}$
<i>lct32</i>	406	TcmL (P16539), 74/64	KS $_{\beta}$
<i>lct33</i>	115	TcmH (P39889), 66/52; ElmH (AAF73051), 64/42	Monooxygenase
<i>lct34</i>	248	G111934 (BAC89875), 51/35	Thioesterase
<i>lct35</i>	322	TcmO (P39896), 61/50	O-Methyltransferase
<i>lct36</i>	429	UrdGT1a (AAF00214), 66/51	Glycosyltransferase
<i>lct37</i>	139	G113466 (BAC91407), 62/40	Hypothetical protein, ester cyclase
<i>lct38</i>	493	SAV1847 (BAC69558), 68/49	Membrane efflux protein
<i>lct39</i>	332	SCO6554 (CAA22358), 61/45	Transcriptional regulatory protein
<i>lct40</i>	252	SCO7810 (CAC03628), 70/55	Oxidoreductase
<i>lct41</i>	558	TcmG (P39888), 63/46; ElmG (AAF73050), 61/46	Dioxygenase
<i>lct42</i>	108	ElmH (AAF73051), 60/38; TcmH (P39889), 57/44	Monooxygenase
<i>lct43</i>	308	SAV4759 (BAC72471), 55/43	Oxidoreductase
<i>lct44</i>	337	LanT (ADD13550), 74/63	NDP-hexose 3-ketoreductase
<i>lct45</i>	468	LanS (AAD13549), 76/70; UrdS (AAF72552), 76/68	NDP-hexose 2,3-dehydratase
<i>lct46</i>	427	UrdQ (AAF72550), 91/80; LanQ (AAD13547), 90/80	NDP-hexose 3-dehydratase
<i>lct47</i>	310	LanZ3 (AAD13561), 56/44; UrdZ3 (AFF72549), 53/44	NDP-hexose 4-ketoreductase
<i>lct48</i>	326	LanH (AAD13546), 84/73; UrdH (AAF00211), 80/67	NDP-hexose 4,6-dehydratase
<i>lct49</i>	355	LanG (AAD13545), 77/65; UrdG (AAF00210), 76/63	NDP-hexose synthetase
<i>lct50</i>	161	LanZ1 (AAD13558), 80/69; UrdZ1 (AAF00208), 71/59	NDP-hexose 3,5-epimerase
<i>lct51</i>	158	Glr0227 (BAC88168), 55/38	Hypothetical protein, ester cyclase
<i>lct52</i>	405	MetK (AAD22464), 91/84	S-Adenosylmethionine synthetase
<i>lct53</i>	542	SCO6284 (CAC37885), 88/80	Probable acyl-CoA decarboxylase
<i>lct54</i>	194	SAV5143 (BAC72855), 69/49	ArsR regulatory protein
<i>lct55</i>	378	SCO3305 (CAB45339), 69/52	Integral membrane acyltransferase
<i>lct56</i>	297	SAV4467 (BAC72179), 90/85	rRNA methyltransferase, lactonamycin resistance?
<i>orf57</i>	322	SAV4466 (BAC72178), 91/82	Putative IS1650 family transposase

^a aa, amino acids.

FIG. 7. Potential role played by lactonamycin genes in biosynthesis of NDP-L-rhodinose.

incorporation of glycine into lactonamycin Z, it is conceivable that Lct28 might transform glycine or a glycine derivative into the corresponding adenylate for use as the starter unit in polyketide assembly (see Discussion). Lct37 and Lct51 exhibit similarities to ester cyclases, while Lct55 exhibits high similarity to a family of membrane-bound acyltransferases. Last, Lct53 appears to encode an acyl CoA decarboxylase. No clear function can be assigned to any of these proteins.

(iii) Genes encoding enzymes for NDP-L-rhodinose biosynthesis. Lactonamycin contains an L-rhodinose moiety, which is attached to the C-5a hydroxyl group of the aglycone. Investigations of the biosynthesis of the aromatic polyketide urdamycin have identified seven genes that are required for NDP-L-rhodinose biosynthesis in *Streptomyces fradiae* (17). Homologs of each of these genes are present in the lactonamycin gene cluster (Table 3). The potential roles played by these genes in the formation of NDP-L-rhodinose are shown in Fig. 7.

(iv) Genes encoding potential regulatory and resistance proteins. The lactonamycin gene cluster contains an unusually large number of genes that display signatures for regulatory proteins. Potential functions can be assigned to three of these proteins, Lct13, Lct14, and Lct15. These three proteins exhibit similarities of 78%, 63%, and 75% to TylP, TylQ, and TylS, respectively. The latter three proteins are part of a regulatory network that controls production of the macrolide antibiotic tylosin in *S. fradiae* (4). In this network, both TylP and TylQ act as repressors, while TylS functions as an activator. The repressor activity of TylP is regulated by the binding of a γ -butyrolactone signaling molecule. A similar function would be anticipated for Lct13. The role assigned to Lct13 is supported by the fact that the proteins encoded by *lct9*, *lct11*, and *lct12* exhibit strong similarities to proteins required for γ -butyrolactone biosynthesis (22, 23). Both Lct15 and TylS show strong similarities to the family of activator proteins, such as ActII-ORF4 and DnrI that are known as SARPs (*Streptomyces* antibiotic regulatory proteins) (56).

The *lct* gene cluster contains two genes whose encoded proteins might provide *S. rishiriensis* with lactonamycin resistance. The first of these proteins is Lct38, which appears to be a member of the

major facilitator superfamily of membrane efflux proteins. A protein in this superfamily has been found to confer resistance to the azoxy antibiotic valanimycin on the producing organism, *Streptomyces viridifaciens* (26). The second candidate for a lactonamycin resistance protein is encoded by *lct56*. Lct56 exhibits high similarity to rRNA methyltransferases. Methylation of specific 23 S rRNA nucleotides is a well-established mechanism for bacterial resistance to macrolide antibiotics (12).

(v) Genes encoding proteins associated with primary metabolism. There are four genes embedded within the *lct* cluster that encode proteins associated with primary metabolism. The proteins encoded by these genes, Lct18, Lct19, Lct20, and Lct52, exhibit high similarities to proteins that are required to regenerate S-adenosyl-L-methionine (AdoMet) from S-adenosylhomocysteine after the transfer of a methyl group from AdoMet. The function of these genes is presumably to supply AdoMet to the methyltransferases Lct25 and Lct35. A similar set of AdoMet biosynthetic genes has been found within the biosynthetic gene cluster of the type I polyketide antibiotic lankamycin (32).

Cloning of the lactonamycin Z biosynthetic gene cluster from *S. sanglieri*. Investigations with the lactonamycin producer *S. rishiriensis* revealed that consistent production of lactonamycin could not be maintained. Fermentations derived either from *S. rishiriensis* spore stocks stored at -80°C or from lyophilized spore stocks gave erratic results. Furthermore, lactonamycin production levels were negatively affected by protoplast formation and by the manipulations required to produce exconjugants. Consequently, investigations of *S. sanglieri* AK 623 were commenced. The production of lactonamycin Z by this organism was found to be more robust than the production of lactonamycin by *S. rishiriensis*. Southern hybridization of the genomic DNA of the *S. sanglieri* AK 623 with probes derived from the *lct33*, *lct36*, and *lct53* genes showed that unique homologs of these genes exist in *S. sanglieri* AK 623, suggesting the existence of a similar gene cluster for lactonamycin Z (data not shown). Therefore, the *lct36* gene of *S. rishiriensis* was amplified by PCR, labeled with ^{32}P , and used to

TABLE 4. Organization and analysis of partial lactonamycin Z gene cluster from *S. sanglieri* AK 623

Gene designation	Size (aa) ^a	Protein homolog(s) [GenBank accession number(s)], % similarity/% identity	Possible function, % identity to <i>S. rishiriensis</i> homolog
<i>lcz24</i>	93	Sim4 (AAL15582), 71/52	Acyl carrier protein, 81
<i>lcz25</i>	335	TcmN (P16599), 66/49	<i>O</i> -Methyltransferase, 83
<i>lcz26</i>	83	RPC_2301 (ABD87855), 70/44	Acyl carrier protein, 69
<i>lcz27</i>	153	RubF (AAG03070), 70/58	Polyketide cyclase, 87
<i>lcz28</i>	524	SAV1250 (BAC68960), 63/51	Carboxylic acid adenylation, 75
<i>lcz29</i>	109	ElmI (AAF73052), 64/46	Polyketide cyclase, 83
<i>lcz30</i>	145	TcmJ (P16558), 70/50	Polyketide cyclase, 90
<i>lcz31</i>	421	TcmK (P16538), 80/67	KS _α , 90
<i>lcz32</i>	406	TcmL (P16539), 74/65	KS _β , 87
<i>lcz33</i>	115	TcmH (P39889), 67/52; ElmH (AAF73051), 64/43	Monooxygenase, 80
<i>lcz34</i>	246	G111934 (BAC89875), 52/37	Thioesterase, 70
<i>lcz35</i>	323	TcmO (P39896), 62/49	<i>O</i> -Methyltransferase, 84
<i>lcz36</i>	427	UrdGT1a (AAF00214), 66/51	Glycosyltransferase, 83
<i>lcz37</i>	137	SAV3997 (BAC71709), 50/34	Hypothetical protein, ester cyclase, 81
<i>lcz38</i>	491	SAV1847 (BAC69558), 65/47	Membrane efflux protein, 81

^a aa, amino acids.

screen *S. sanglieri* cosmid libraries constructed in pOJ446 and SuperCos1. A total of 38 positive pOJ446-derived cosmids and 17 positive SuperCos1-derived cosmids were isolated. One pOJ446-derived cosmid, pCAK7, was initially chosen for sequencing, since it also exhibited strong hybridization to probes derived from the *lct33* and *lct53* genes (data not shown). Sequencing of ca. 13 kb of DNA from the insert in cosmid pCAK7 disclosed the presence of 15 ORFs. These ORFs are listed in Table 4. All 15 ORFs in the sequenced region of *S. sanglieri* DNA exhibit high similarities of genes found in the *lct* cluster. Furthermore, the organization of these genes is identical to that found in the *lct* cluster. These findings provide strong evidence that both the *S. rishiriensis* and *S. sanglieri* clusters are involved in the production of similar metabolites. The functions of the 15 *lcz* genes are expected to be similar or identical to those of the corresponding *lct* genes.

Confirmation that the cloned locus from *S. sanglieri* is required for lactonamycin Z production. To confirm that the cloned locus from *S. sanglieri* contains genes that are required for lactonamycin Z production, a double-crossover gene disruption was created in the glycosyltransferase gene *lcz36*. The disruption cosmid, pKOlcz36, was created by replacement of nt 40 to 1281 of *lcz36* in cosmid pCAS2 with the *aac(3)IV-oriT* cassette of pIJ773 using lambda Red-mediated recombination (15, 16). Introduction of cosmid pKOlcz36 into *S. sanglieri* by intergeneric conjugation led to the desired disruptant, as judged by PCR amplification of the disrupted region and restriction digests of the resulting PCR product (Fig. 2) as well as by Southern hybridization experiments (Fig. 3). The $\Delta lcz36$ mutant was cultured under standard conditions, and the combined organic extracts of the broth and mycelium were analyzed by HPLC. The HPLC analysis (Fig. 4) showed the absence of lactonamycin Z. A complementation construct, pOElcz36, was generated by placing the *ermEp** promoter in front of the *lcz36* gene cloned into plasmid pXZ152. Plasmid pXZ152 was derived from the integrating plasmid pSET152 by introduction of the thiostrepton resistance gene of pIJ702. The complementation construct was introduced into the $\Delta lcz36$ mutant by intergeneric conjugation. Restoration of lactonamycin Z production was confirmed by HPLC analysis (Fig. 4) and by

isolation and NMR characterization of lactonamycin Z from the complemented strain (data not shown).

DISCUSSION

PCR primers designed to conserved regions of NDP-hexose 2,3-dehydratase genes were used to amplify a 2,3-dehydratase DNA probe from the lactonamycin producer *S. rishiriensis*. This probe was used to clone a gene cluster from *S. rishiriensis* whose sequence analysis suggested it was likely to be the lactonamycin biosynthetic gene cluster of this organism. Unfortunately, lactonamycin production by this organism was readily lost during the course of genetic manipulations. This behavior created major obstacles to the interpretation of gene disruption experiments. For this reason, investigations of the lactonamycin Z producer, *S. sanglieri*, were initiated. The lactonamycin Z gene cluster was cloned from this organism by using the glycosyltransferase gene *lct36* from the lactonamycin gene cluster as a probe. Sequencing of ca. 13 kb of the cloned *S. sanglieri* DNA disclosed the presence of 15 ORFs. All 15 of these genes exhibit high similarity to genes within the *S. rishiriensis* cluster as well as an identical organization. This suggests that both gene clusters are coding for the production of similar metabolites in both organisms. Unequivocal evidence that the cloned *S. sanglieri* genes are required for lactonamycin Z biosynthesis was obtained by inactivation of the glycosyltransferase gene *lcz36* by means of a double-crossover gene disruption and by complementation of the resulting mutation. HPLC analysis of the spectrum of metabolites produced by the *lcz36* disruptant revealed the absence of lactonamycin Z (Fig. 4). Introduction of an intact copy of *lcz36* under the control of the *ermEp** promoter into the *lcz36* disruptant restored production of lactonamycin Z (Fig. 4). These results confirm that the genetic locus cloned from *S. sanglieri* is required for lactonamycin Z production, and they support the hypothesis that the highly similar locus cloned from *S. rishiriensis* is necessary for lactonamycin production. Although the entire lactonamycin Z gene cluster of *S. sanglieri* has not been fully sequenced, the more extensive sequencing of the *S. rishiriensis* gene cluster appears to have reached the lactonamycin cluster boundaries. The 5'

end of the lactonamycin gene cluster probably begins with *lct8*, since the ORF immediately upstream appears to encode S-adenosylmethionine decarboxylase. The 3' end of the lactonamycin cluster is probably defined by *lct56*, since the ORF immediately downstream exhibits a high degree of similarity to the IS1650 family of transposases.

Important insights into the biosynthesis of the aglycone core, lactonamycinone, were obtained from precursor incorporation experiments. Administration of sodium [1-¹³C]-, [2-¹³C]-, and [1,2-¹³C₂]acetate to *S. sanglieri* produced labeling patterns that are consistent with the head-to-tail incorporation of nine acetate units into lactonamycinone (Table 2). The ¹³C-labeling pattern observed for rings E and F of lactonamycinone derived from the labeled forms of acetate can be rationalized by the oxidative cleavage of ring D of a naphthacenequinone intermediate similar to TcmD3 (Fig. 5). Most significantly, no labeling of C-12 and C-12a was produced in any of these incorporation experiments. This observation suggested that the polyketide assembly process is initiated by use of an alternative starter unit. This hypothesis was confirmed by administration of [1,2-¹³C₂]-, [1-¹³C]-, and [2-¹³C, ¹⁵N]glycine to *S. sanglieri*. The labeling patterns observed in lactonamycin Z derived from these precursors indicate that the nitrogen atom and both carbon atoms of glycine serve as the source of the polyketide starter unit (Table 2). The use of alternate starter units in type II aromatic polyketide assembly is rare, but not unprecedented. For example, propionate is used as the starter unit for daunorubicin biosynthesis in *S. peucetius* (3, 39), several short-chain carboxylic acids serve as starter units for the anthroquinones R1128A-D produced by *Streptomyces* sp. strain R1128 (28), and benzoate functions as the starter unit for enterocin in *Streptomyces maritimus* (20). The mechanism involved in the incorporation of alternative starter units varies in each of these examples (33). The lactonamycin and lactonamycin Z gene clusters exhibit several novel features that may provide a clue to the mechanism of glycine incorporation. One of these features is the presence of genes (*lct28* and *lcz28*) that encode a protein with high similarities to acyl-CoA synthetases and carboxyl adenylation enzymes. Recent studies have identified the amino acid residues that determine the substrate specificity of enzymes or domains that catalyze the adenylation of α -amino acids (8, 48). Analysis of Lct28 and Lcz28 reveals that the specificity codes for these proteins are DLFVLCLL and DLFILCLL, respectively. Since the consensus specificity code for glycine is DILQ(L/V)G(L/M/V)I (9, 31), it appears unlikely that glycine serves as a substrate for either Lct28 or Lcz28. An alternative substrate could be sarcosine (*N*-methylglycine), for which a specificity code has not been clearly defined. Another novel feature of the *lct* and *lcz* gene clusters is the presence of two genes in each cluster that encode ACPs. Of these ACPs, only Lct24 and Lcz24 exhibit strong similarity to the ACPs of other type II PKSs. This suggests that the other ACPs, Lct26 and Lcz26, may play a role in the initiation of lactonamycinone biosynthesis with a glycine derivative. This hypothesis is supported by the fact that both the R1128 and frenolicin gene clusters contain an additional ACP that functions to initiate polyketide synthesis with an alternative starter unit (52). Therefore, it appears that initial stages of lactonamycinone biosynthesis may involve activation of a glycine derivative by Lct28 or Lcz28, attachment of the activated amino acid to Lct26 or Lcz26 as a thioester, and transfer of the amino acid from Lct26 or Lcz26 to the ketosynthase Lct31 or Lcz31 by thioester exchange.

A third unusual feature of the lactonamycin gene clusters is the presence of a thioesterase (Lct34 and Lcz34) in each cluster. Although thioesterase domains and stand-alone thioesterases are often found in association with type I polyketide synthases, the occurrence of thioesterases within type II polyketide gene clusters is very rare (32). Recent studies of the acyltransferase homolog ZhuC found within the R1128 PKS have shown that ZhuC exhibits thioesterase activity and selectively hydrolyzes acetyl-ACPs formed by malonyl-ACP decarboxylation (51). ZhuC therefore appears to function as an editing enzyme to remove acetate starter units that might otherwise compete with the alkyl acyl starter units. Investigations by Bisang et al. (6) have shown that the decarboxylation of malonyl-ACPs is catalyzed by the KS _{β} (chain length factor) of the minimal PKS and to a lesser extent by the KS _{α} . The ability of the KS _{β} protein to catalyze malonyl-ACP decarboxylation is dependent upon the presence of a conserved glutamine residue. It appears likely that the KS _{β} proteins in the *lct* and *lcz* clusters can catalyze malonyl-ACP decarboxylation, since the conserved glutamine residue is present in the amino acid sequences of both proteins. For this reason, it is conceivable that the thioesterase encoded in each cluster may function in a similar manner to ZhuC in order to prevent initiation of lactonamycinone biosynthesis with acetate. Additional investigations will clearly be required to elucidate the details of glycine incorporation into lactonamycinone.

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