Characterization of the Biosynthesis Gene Cluster for the Pyrrole Polyether Antibiotic Calcimycin (A23187) in *Streptomyces chartreusis* NRRL 3882

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The pyrrole polyether antibiotic calcimycin (A23187) is a rare ionophore that is specific for divalent cations. It is widely used as a biochemical and pharmacological tool because of its multiple, unique biological effects. Here we report on the cloning, sequencing, and mutational analysis of the 64-kb biosynthetic gene cluster from *Streptomyces chartreusis* NRRL 3882. Gene replacements confirmed the identity of the gene cluster, and *in silico* analysis of the DNA sequence revealed 27 potential genes, including 3 genes for the biosynthesis of the α-ketopyrrole moiety, 5 genes that encode modular type I polyketide synthases for the biosynthesis of the spiroketal ring, 4 genes for the biosynthesis of 3-hydroxyantranilic acid, an N-methyltransferase tailoring gene, a resistance gene, a type II thioesterase gene, 3 regulatory genes, 4 genes with other functions, and 5 genes of unknown function. We propose a pathway for the biosynthesis of calcimycin and assign the genes to the biosynthesis steps. Our findings set the stage for producing much desired calcimycin derivatives using genetic modification instead of chemical synthesis.

Calcimycin (A23187) is one of few natural ionophore antibiotics that specifically transport divalent cations such as calcium and magnesium (25). Calcimycin inhibits the growth of Gram-positive bacteria and some fungi (20). It also inhibits the activity of ATPase and uncouples oxidative phosphorylation of mammalian cells, and it induces apoptosis of cultured cells via direct activation of intracellular signals (17, 29). Calcimycin is widely used as a biochemical tool for pharmacological and *in vitro* toxicological studies, and it has been mentioned in more than 16,000 publications since its discovery in 1972.

There are two additional natural pyrrole polyether antibiotics similar in structure to calcimycin: X-14885A from *Streptomyces chartreusis* (NRRL 12350) and cezomycin (8, 19, 35). Cezomycin is also produced by *S. chartreusis* NRRL 3882 as a precursor for the much more valuable calcimycin (8). All three antibiotics feature an α-ketopyrrole, a substituted benzoxazole, and a spiroketal ring. They differ only in the nature of two side groups (Fig. 1).

Calcimycin and a number of similar molecules have been chemically synthesized (26–28). Calcimycin derivatives have also been generated by microbial transformation (1). It would, however, be desirable to produce additional calcimycin derivatives by fermentation using genetically modified bacteria. This requires targeted mutation of the sequenced antibiotic biosynthesis gene cluster.

The cloning of the calcimycin biosynthetic genes was greatly aided by the data from feeding experiment using isotopes and nuclear magnetic resonance analysis, which indicated that the α-ketopyrrole is derived from L-proline, the spiroketal ring is a polyketide derived from propionate and acetate, the benzoxazole is synthesized from glucose via a shikimate-type pathway that produces 3-hydroxyantranilic acid, and the N-methyl group of the benzoxazole comes from methionine (7, 39).

Here we report on the cloning and sequencing of a 64-kb gene cluster that contains genes for the synthesis of the three parts of the calcimycin backbone (benzoxazole, spiroketal ring, and α-ketopyrrole), a modification gene, a resistance gene (to prevent the killing of the producer strain), a type II thioesterase gene, three regulatory genes, four genes with other functions, and five genes of unknown function.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown at 37°C in liquid Luria-Bertani medium or on solid Luria-Bertani medium containing 2% agar, as described by Sambrook and Russell (31). *S. chartreusis* NRRL 3882 was cultured at 30°C in tryptic soy broth and yeast extract (TSBY) medium for the isolation of chromosomal DNA and on solid mannitol soy flour (SFM) medium for spore collection and conjugation (15).

The antibiotics ampicillin (100 μg/ml), apramycin (30 μg/ml), chloramphenicol (25 μg/ml), and kanamycin (50 μg/ml) were used for the selection of recombinants. DNA isolation, manipulation, and plasmid construction. DNA isolation and manipulation were performed as described by Sambrook and Russell (31). DNA fragments were recovered from agarose gels using a Tiangen purification kit (Beijing, China), according to the supplied protocol. Restriction endonucleases and other enzymes were purchased from NEB Co. Ltd. Enzyme reactions were carried out according to the instructions provided by the manufacturer. Synthetic primers were purchased from Sangon Corp. (Shanghai, China). Clones were sequenced by Invitrogen Corp. (Shanghai, China).

Cloning and sequencing of calcimycin biosynthetic gene cluster. (i) **PCR amplification of the proline adenyltransferase gene from *S. chartreusis* NRRL 3882.** Fifteen sets of degenerate primers were designed on the basis of the conserved domains of bacterial proline adenyltransferase genes found in pyruvate, dicarboxylate, and lactate dehydrogenases.
teorin, coumermycin A₁, prodiginines, clorobiocin, and pyrrolomycin biosynthetic gene clusters (5, 23, 24, 34, 37). PCR amplification was run for 30 cycles. The conditions for each cycle were 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C. Four different PCR fragments were purified after agarose gel separation and cloned into the PMD 18-T vector (TaKaRa Corp.) for sequencing. One pair of successful primers was QL1-FP1 and QL1-RP3 (Table 2).

(ii) Construction and screening of a cosmid library. The *S. chartreusis* NRRL 3882 cosmid library was constructed using a CopyControl fosmid library production kit, according to the protocol provided by the company. A pair of nondegenerate O2L2 primers (O2L2-FP1 and O2L2-RP1) were designed according to the above sequencing result, and the labeled PCR product was then used to screen the cosmid library (Fig. 2 and 3; Table 2).

PCR primers QL7P1 (QL7-FP1 and QL7-RP1) and QL7P3 (QL7-FP3 and QL7-RP3) were then designed to amplify sequences near the two ends of cosmid p16F9 (Table 2). The labeled PCR products were then used to identify flanking cosmids, such as p6F5 and p14F11 (Fig. 3). Sets of cosmids that hybridized to one of the PCR probes were digested using PstI. The large numbers of cosmids digests that were compared made it possible to deduce the PstI restriction map of the entire contig. This, in turn, made it possible to select the two cosmids p6F5 and p14F11 that overlapped the central cosmid p16F9 only a short distance. The three cosmids were sequenced, and the sequences were aligned using BioEdit software, version 4.8.10. Open reading frames (ORFs) were predicted using FramePlot software, version 4.0 (http://nocardia.nih.go.jp/fp/4). The putative proteins were compared with data online using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Module and domain organizations of polyketide synthases (PKSs) were deduced by NRPS-PKS software online (http://www.nri.res.in/nrps-pks.html) (2, 36).

gene inactivation and complementation. The targeted genes from the calcimycin gene cluster of *S. chartreusis* NRRL 3882 were disrupted using the Redirct technology, according to the supplied protocol, with some modifications (12). Briefly, the neo gene from SuperCos1 was used to replace an internal region of the target gene. For the construction of the calN2 disruption plasmid, a 3,679-bp BamHI fragment encoding calN2 from cosmid p16F9 was cloned into the BamHI

![FIG. 1. Structures of calcimycin, cezomycin, and X-14885A.](http://aac.asm.org/content/55/1/976.full)

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>Streptomyces chartreusis</em></td>
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<tr>
<td>NRRL 3882</td>
<td>Wide type, calcimycin producer</td>
<td>NRRL</td>
</tr>
<tr>
<td>WQL1</td>
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</tr>
<tr>
<td>WQL2</td>
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</tr>
<tr>
<td>WQL4</td>
<td>ΔcalB2, calcimycin negative</td>
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<tr>
<td>WQL5</td>
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<tr>
<td>BW25113(pIJ790)</td>
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<td>CopyControl fosmid library production kit</td>
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<th><strong>Plasmids</strong></th>
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<td>Stratagene</td>
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<tr>
<td>pOJ446</td>
<td>aac(3)IV ori (SCP2*) rep trim ori cos</td>
<td>3</td>
</tr>
<tr>
<td>pSET152</td>
<td>aac(3)IV lacZ rep trim attC31 ori T</td>
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<td>pUC18 derivative T vector</td>
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<td>pOJ446-derived plasmid carrying calN2</td>
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</tr>
<tr>
<td>pJTU3170</td>
<td>pJTU3167-derived plasmid, neo, calN2-defective plasmid</td>
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<td>pJTU3171</td>
<td>pOJ446-derived plasmid carrying calN1 and calB2</td>
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<td>pJTU3178</td>
<td>pJTU3171-derived plasmid carrying neo resistance gene, calB1-defective plasmid</td>
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</tr>
<tr>
<td>pJTU3185</td>
<td>pSET152-derived plasmid carrying calN2, ΔcalN2 complementation plasmid</td>
<td>This work</td>
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* Cml, chloramphenicol resistance; Kan, kanamycin resistance; aac(3)IV, apramycin resistance; neo, kanamycin resistance.

<table>
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<th>Table 2. Primers used in this study</th>
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</tr>
<tr>
<td>Q1L2-RP1</td>
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<tr>
<td>Q1L7-FP1</td>
</tr>
<tr>
<td>Q1L7-RP1</td>
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<tr>
<td>Q1L7-RP3</td>
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* N is A, T, G, or C; S is G or C; Y is C or T; and R is A or G.
site of pOJ446 to produce pJTU3167. For the construction of the calN1 and calB2 disruption plasmids, a 7,889-bp PstI fragment harboring calN1 and calB2 from cosmid p16F9 was cloned into the PstI site of pBluescript SK(+), generating pJTU3157. The 7,889-bp SpeI/EcoRV fragment from pJTU3157 was cloned into the SpeI/EcoRV sites of pOJ446 to yield pJTU3171. The neo gene, amplified using KOD-plus DNA polymerase (Toyobo), was introduced into pJTU3167 and pJTU3171 using the PCR targeted method, generating the desired plasmids, pJTU3170 (calN2), pJTU3175 (calN1), and pJTU3178 (calB2). These mutant plasmids were then introduced into S. chartreusis NRRL 3882 by conjugation from E. coli ET12567/pUZ8002. Apramycin-sensitive and kanamycin-resistant colonies were isolated as WQL1 (calN2), WQL2 (calN1), and WQL4 (calB2). Successful gene disruptions were confirmed by PCR and high-pressure liquid chromatography (HPLC) and mass spectrometric (MS) analysis (Fig. 4 to 6).

To construct the plasmid for gene complementation of calN2, a BamHI-digested fragment carrying calN2 (from pJTU3166) was cloned into the BamHI site of pSET152, generating pJTU3185. Introduction of pJTU3185 into WQL1 (calN2) via conjugation generated the complemented strain, WQL2.

HPLC and MS analysis of calcimycin biosynthesis. S. chartreusis NRRL 3882 and its mutant derivatives were cultivated at 30°C for 6 to 10 days with SFM medium. Then, the culture medium was extracted with an equal volume of methanol. The resulting extracts were concentrated in vacuo and redissolved in methanol for HPLC/MS analysis using an Agilent 1100 series LC/MSD Trap system and an Agilent Zorbax SB-C18 (2.1 by 50 mm) column. HPLC was performed with a linear gradient of 60% of 85% CH3OH-H2O (0.1% trifluoroacetic acid) over 30 min at a flow rate of 0.2 ml/min and monitoring at 280 nm. The calcimycin standard, purchased from Sigma-Aldrich, was used as a control.

Nucleotide sequence accession number. The nucleotide sequence obtained in this study was submitted to GenBank database, under accession no. HM452329.

RESULTS

Cloning and sequencing of calcimycin biosynthetic gene cluster. Calcimycin contains three distinctive structures: a spiroketal ring, a benzoxazole heterocycle, and an α-ketopyrrole moiety (Fig. 1). Of these, only α-ketopyrrole moieties are found in a wide variety of biologically active natural products. Feeding experiments have shown that the α-ketopyrrole of calcimycin is derived from L-proline (11, 33). Biosynthesis of...
α-ketopyrrole starts with the activation of L-proline to L-prolyl-AMP by a specific adenyltransfease. Aligning the amino acid sequences of six adenyltransferases involved in the biosynthesis of five different antibiotics identified four conserved regions (Fig. 2). We synthesized degenerate primers according to these conserved sequences, and using *S. chartreusis* genomic DNA as a template, we obtained a PCR fragment of the expected size of 460 bp. The sequence of the DNA fragment was consistent with it encoding a *S. chartreusis* L-proline adenyltransferase (CalN2 in Fig. 3). We then used the labeled 460-bp DNA fragment as a hybridization probe to identify *S. chartreusis* cosmid clones that contained parts of the calcimycin biosynthesis gene cluster.

Several hybridizing cosmids were isolated and positioned relative to each other according to their PstI restriction maps (Fig. 3). All the cosmids formed a single ca. 64-kb contig. One of the central cosmids, p16F9, was sequenced. It contained on both ends incomplete type I PKS genes which were likely to be involved in the biosynthesis of calcimycin.

To find flanking cosmids that overlapped p16F9 only by a short sequence, PCR probes QL7P1 and QL7P3 (Fig. 3A) were generated from either end for chromosome walking. PstI restriction mapping identified cosmid p6F5 and p14F11, which extend far on either side of p16F9. Together, the three contiguous cosmids cover a 101-kb region with an overall G+C content of 72.0%, which is normal for *Streptomyces* (the sequence of the 64-kb region shown schematically in Fig. 3B has been deposited in GenBank under accession no. HM452329).

Translated open reading frames were identified using FramePlot, and database searches identified 27 clustered ORFs spanning a 64-kb DNA region. We chose the designation *cal* (calcimycin) for all these putative antibiotic biosynthesis genes (Fig. 3B; Table 3).

Deletion of *calN1*, *calN2*, and *calB2* proved that these genes are required for calcimycin biosynthesis. We predicted that *calN1* (acyl coenzyme A [acyl-CoA] dehydrogenase) and *calN2* (L-proline adenyltransferase) are required for the biosynthesis of the α-ketopyrrole structure and that *calB2*, isochorismatase, may be required for the synthesis of the 3-hydroxyanthranilic acid moiety. The three genes were individually deleted from the calcimycin producer using gene replacements, as shown
in Fig. 4 to 6. The resulting mutant strains were tested for antibiotic production using HPLC/MS. As expected, deletion of each of these three genes abolished the production of calcimycin.

To make sure that the lack of antibiotic production was indeed caused by the gene replacement rather than by a spontaneous mutation elsewhere in the S. chartreusis genome, we reintroduced caln2 cloned onto a plasmid, pSET152, into the strain from which this gene had been removed. This trans complementation restored calcimycin production and thus proved that caln2 is required for calcimycin production (Fig. 4).

In silico analysis of the calcimycin gene cluster. (i) Pyrrole moiety biosynthesis. The putative proteins CalN1 to CalN3 are most similar to CloN3 to CloN5, respectively (61%, 60%, and 48% amino acid identities, respectively), of Streptomyces roseochromogenes DS 12.976, which have been proved to activate chorismate. The second double bond might form spontaneously by air oxidation. The active-site cysteine residue of the KS domain of module 1 to prime the biosynthesis of the polyketide chain. Finally, CalG resembles type II thioesterases (TEs) and probably releases the completed polyketide from the PKS, and it may also serve as an editing enzyme by cleaving miscognate chains from the ACP domains (9, 13, 16, 38).

(ii) Polyketide chain biosynthesis. The noncontiguous genes calA1 to calA5 encode five noncontiguous type I multifunctional, modular PKSs (Fig. 3B; Table 3). Each PKS module should minimally contain a ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP). Optionally, there are also a dehydratase (DH), an enoyl reductase (ER), and a β-ketoreductase (KR). Missing optional activities may have inactivating point mutations, or the entire domain may be deleted. The optional domains determine the reduction state of the incorporated extender unit, which can be a keto group, a hydroxyl group, a trans double bond, or a saturated CH2.

The NRPS-PKS online software was used to deduce the functionalities of the five CalA PKSs containing seven PKS modules (Table 4) (2, 36). Each module has a different predicted functionality which made it easy to assign the modules unambiguously to the sequence of polyketide synthesis predicted for calcimycin (Fig. 8, structure 3, which shows the active-site cysteine residue of the KS domain of module 1 to prime the biosynthesis of the polyketide chain. Finally, CalG resembles type II thioesterases (TEs) and probably releases the completed polyketide from the PKS, and it may also serve as an editing enzyme by cleaving miscognate chains from the ACP domains (9, 13, 16, 38).

(iii) 3-Hydroxyanthranilic acid biosynthesis. Four genes (calB1 through calB4) within the cal gene cluster encode pro-
The proposed gene assignment for the pathway is shown in Fig. 7C and Table 3. The shaded circles indicate catalytic centers (domains): KS, AT (p, methylmalonyl-CoA specific; a, malonyl-CoA specific), DH, KR, ER, and ACP. The black arrows indicate multidomain PKS synthases containing one or two PKS modules. The black arrows denote reactions that are not predicted to be catalyzed by any of the genes shown in Fig. 3. Putative functions of the genes: CalB1, anthranilate synthase; CalB2, isochorismatase; CalB3, 3-deoxy-3-arabino-heptulosonate 7-phosphate synthase; CalB4, 3-deoxy-3-arabino-heptulosonate 7-phosphate synthase. Compounds: PEP, phosphoenolpyruvate; E4P, erythrose-4-phosphate; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate; ADIC, 2-amino-2-deoxyisochorismate; DHHA, trans-2,3-dihydro-3-hydroxyanthranilic acid; HA, 3-hydroxyanthranilic acid.

DISCUSSION

Three gene replacement experiments and the predicted functions of 13 putative proteins, including 6 multifunctional PKS modules, indicate that we have cloned and sequenced the gene cluster coding for the biosynthesis of the very important divalent ionophore calcimycin.

Genes required for the biosynthesis of the pyrrole, spiroketal polyketide, and benzoxazole moieties (Fig. 1) have been clearly identified by the high similarities of the predicted proteins to known enzymes from other antibiotic biosynthesis pathways,
and their functions are consistent with the results of precursor feeding experiments (39).

Four peripheral genes (\textit{calC}, \textit{calD}, \textit{calF}, \textit{calH}) have extensive end-to-end similarities to enzymes of known functions, but it is not certain that they are involved in calcimycin biosynthesis. The genes \textit{cal1} and \textit{cal29} are very similar to peptidases S15 and M20, respectively. Both genes are probably irrelevant to calcimycin production. It therefore seems likely that all the

\begin{table}[h]
\centering
\caption{PKSs organization in the calcimycin biosynthetic gene cluster}
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
\textbf{CalA} & \textbf{Order} & \textbf{PKS domain in the order of the sequence} & \textbf{Predicted} & \\
\textbf{module} & \textbf{use} & \textbf{KS (3)} & \textbf{AT} & \textbf{DH} & \textbf{ER} & \textbf{ACP} & \\
\hline
1a & 1 & 1 & 1 & Not present & Not present & Not present & C=O, CH\textsubscript{3} \\
1b & 2 & 2 & 2 & Not present & Not present & Not present & CHOH, CH\textsubscript{3} \\
2 & 3 & 3 & 3 & 3 HXXXGXXXXXP & 3 & 3 K-S-Y & CH\textsubscript{3}, CH\textsubscript{2} \\
5a & 4 & 4 & 4 & Inactive, HXXXGXXXXXP & Not present & Inactive, R-S-L & C=O \\
5b & 5 & 5 & 5 & 5 HXXXGXXXXXP & 5 & 5 K-S-Y & CH\textsubscript{3}, CH\textsubscript{2} \\
4 & 6 & 6 & 6 & Not present & Not present & 6 (C-10) K-S-Y & CHOH \\
3 & ? & Present & Inactive, S \rightarrow G & Not present & Not present & No chain elongation & \\
\hline
\end{tabular}
\footnotesize{\textsuperscript{a} A bold number means that the indicated functional domain is predicted to be active. The numbers in parentheses in the subheads refer to those in the order-of-use column. \\
\textsuperscript{b} a, malonate is used to incorporate acetate; p, methylmalonate is used to incorporate propionate. Active AT domains feature the conserved catalytic key residue Ser. \\
\textsuperscript{c} Functional DH domains have the conserved motif HXXXGXXXXXP. The mutated conserved amino acids are printed in boldface. \\
\textsuperscript{d} Functional ER domains feature the conserved motif GXGXXGXXXA. \\
\textsuperscript{e} Functional KR domains contain the conserved consensus motif GXGXXGXXXA associated with the NADP(H) binding site and the K-S-Y catalytic triad. The letters 0 and 1 after the module number indicate the predicted stereospecific configuration at the \(\beta\) position of chiral centers, as described previously (30). Note that a chiral center is formed only when the KR is active and the ER and DH are inactive or missing. The number of the C atom is indicated in parentheses, as in Fig. 1. In CalA3, the KR GXGXXGXXXA motif is changed to GXGXXGXXXA. The mutated conserved amino acids are printed in boldface. \\
\textsuperscript{f} Redox state of the \(\beta\)-carbon atom; CH\textsubscript{2}, propionate instead of acetate.}
\end{table}

and 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Proposed pathway for the generation of the benzoxazole ring and further maturation of the calcimycin molecule. The amino group of 3-hydroxyanthranilic acid nucleophilically attacks the terminal carbonyl group of the polyketide chain (substance 3) and releases the polyketide chain (substance 4) from the ACP of the PKS module 6 (Fig. 7). An acetalization reaction then closes the heterocycle ring and generates the benzoxazole moiety (substance 5). Spiroketal ring formation (structure 6) may proceed spontaneously to give cezomycin (substance 2). Hydroxylation (substance 7), amination (substance 8), and N-methylation (catalyzed by CalM) finally generate calcimycin (substance 1). Note that only substances 1 and 2 have actually been observed. The gray background indicates where the last change in the reaction series has taken place. CalD and CalG are only tentatively assigned to the respective reactions.}
\end{figure}
calcimycin biosynthetic genes are within the 64-kb gene cluster shown in Fig. 3 and almost certainly within the 100 kb spanned by the three cosmids clones.

**Polyketide synthesis.** The five PKS genes of the cluster are not contiguous, and they are not arranged in the order of the biosynthetic steps. The program NRPS-PKS predicted that all the domains of CalA3 are deleted or inactive, and its role in calcimycin production remains unclear (2, 36). The remaining six PKS modules have five different predicted functions, giving unambiguous assignment functions to the steps of polyketide synthesis (Table 4). The predicted functions of the PKS modules thus fit exactly the chemical structure of calcimycin. This includes the selection of methylmalonate or malonate by the ACP modules, the redox state of each extender module, and the stereospecificity of the two chiral β-carbon groups, which is determined by the KR modules (Table 4). Both CalA2 and CalA5b (b indicates the second module of the PKS) were predicted to incorporate fully reduced propionate units into the growing polyketide chain (Table 4). Assuming that Cal5b was likely to be used after Cal5a, we tentatively named Cal5a PKS module 4, and Cal5b became PKS module 5 (Table 4). After 6 rounds of decarboxylative condensation using two malonyl-CoA moieties and four methylmalonyl-CoA moieties as extender units, the full-length polyketide chain must be detached from the final module of the assembly line.

A variety of termination strategies for PKS assembly lines are known (9). Most often, a C-terminal PKS TE domain catalyzes the release of the mature polyketide chain. Alternatively, the polyketide chain can be released by a separate type II TE, as exemplified by NanE for nanchangmycin, MonCII for monensin, and NigII for nigericin biosynthesis (9). The polyketide chain can also be released by a reductase domain or other discrete enzymes, such as pyridoxal-phosphate (PLP)-dependent 2-oxoamine synthase, acetyltransferase, lactamase, or Baeyer-Villiger monooxygenase (9).

There is no TE domain at the C-terminal end of CalA4. Instead, we found CalG, a type II TE in the cal gene cluster. CalG may cleave the mature linear polyketide from CalA4 or, more efficiently, it may catalyze directly the intermolecular amide linkage between the amino group of 3-hydroxyanthranilic acid and the polyketide chain (Fig. 8, structure 3).

**Synthesis of benzoxazole moiety.** Precursor feeding experiments showed that 3-hydroxyanthranilic acid is incorporated into the benzoxazole moiety (39). CalB1 to CalB4 are responsible for the biosynthesis of 3-hydroxyanthranilic acid. The extensive similarities of CalB4, CalB1, and CalB2 and PhzC, PhzE, and PhzD, respectively, suggest that this part of the calcinycin pathway is similar to the phenazine biosynthesis. After 6 rounds of decarboxylative condensation using two malonyl-CoA moieties and four methylmalonyl-CoA moieties as extender units, the full-length polyketide chain must be detached from the final module of the assembly line.

**Formation of spirotetal ring.** Spirotetal rings are found in tautomycin, avermectins, and spirandienes (10, 14, 18). It have been postulated that the spirotetal ring formation could proceed spontaneously by dual nucleophilic attack on the carbonyl carbon atom (C-14) by the two hydroxyl groups at C-10 and C-18 (Fig. 8, structure 6).

**Tailoring steps.** Subsequent tailoring steps include hydroxylation, ligation at C-3, and N-methylation of the benzoxazole moiety. N-methylation tailoring is catalyzed by CalM (Q. Wu, submitted). Removal of calM resulted in the accumulation of the calcimycin precursor (Fig. 8, structure 8). This proved that CalM was involved in post-PKS modification of cezomycin.

**Regulation and resistance.** CalR1 and CalR2 resemble TyR-type and LuxR-type transcription regulators, respectively. Their location within the calcimycin gene cluster suggests that they participate in the regulation of calcimycin biosynthesis. The divergently transcribed genes calT and calR probably encode a calcimycin export pump and its regulator. They may also make the producer strain resistant to calcimycin.

**Conclusions.** Calcimycin is special because of its biological activity and because it features a rare benzoxazole moiety. The identification and cloning the cal biosynthetic gene cluster of S. chartreusis NRRL 3882 make it possible to plan the generation of calcimycin derivatives by genetic engineering involving gene replacements and expression of the entire gene cluster in different hosts.

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### REFERENCES
