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ENZYMOLOGY AND PROTEIN ENGINEERING

Cezomycin Is Activated by CalC to Its Ester Form for Further Biosynthesis Steps in the Production of Calcimycin in *Streptomyces chartreusis* NRRL 3882

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Hao Wu,[a] Jingdan Liang,[a] Jialiang Wang,[a] Wei-Jun Liang,[b] Lixia Gou,[c] Qiulin Wu,[a] Xiufen Zhou,[a] Ian J. Bruce,[d] Zixin Deng,[a] and Zhijun Wang[a]

[a]State Key Laboratory of Microbial Metabolism, Joint International Laboratory on Metabolic & Developmental Sciences, School of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai, China

[b]Department of Life and Environmental Sciences, Faculty of Science and Technology, Bournemouth University, Talbot Campus, Fern Barrow, Poole, Dorset, United Kingdom

[c]College of Life Science, North China University of Science and Technology, Tangshan, Hebei, China

[d]School of Physical Sciences, University of Kent, Canterbury, Kent, United Kingdom

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ABSTRACT Calcimycin, N-demethyl calcimycin, and cezomycin are polyether divalent cation ionophore secondary metabolites produced by *Streptomyces chartreusis*. A thorough understanding of the organization of their encoding genes, biosynthetic pathway(s), and cation specificities is vitally important for their efficient future production and therapeutic use. So far, this has been lacking, as well as information concerning any biosynthetic relationships that may exist between calcimycin and cezomycin. In this study, we observed that when a Cal$^-$ (*calB1* mutant) derivative of a calcimycin-producing strain of *S. chartreusis* (NRRL 3882) was grown on cezomycin, calcimycin production was restored. This suggested that calcimycin synthesis may have resulted from postsynthetic modification of cezomycin rather than from a *de novo* process through a novel and independent biosynthetic mechanism. Systematic screening of a number of Cal$^-$ *S. chartreusis* mutants lacking the ability to convert cezomycin to calcimycin allowed the identification of a gene, provisionally named *calC*, which was involved in the conversion step. Molecular cloning and heterologous expression of the CalC protein along with its purification to homogeneity and negative-staining electron microscopy allowed the determination of its apparent molecular weight, oligomeric forms in solution, and activity. These experiments allowed us to confirm that the protein possessed ATP pyrophosphatase activity and was capable of ligating coenzyme A (CoA) with cezomycin but not 3-hydroxyanthranilic acid. The CalC protein's apparent K_m and k_{cat} for cezomycin were observed to be 190 μM and 3.98 min^{-1}, respectively, and it possessed the oligomeric form in solution. Our results unequivocally show that cezomycin is postsynthetically modified to calcimycin by the CalC protein through its activation of cezomycin to a CoA ester form.

IMPORTANCE Calcimycin is a secondary metabolite divalent cation-ionophore that has been studied in the context of human health. However, detail is lacking with respect to both calcimycin's biosynthesis and its biochemical/biophysical properties as well as information regarding its, and its analogues', divalent cation binding specificities and other activities. Such knowledge would be useful in understanding how calcimycin and related compounds may be effective in modifying the calcium channel ion flux and might be useful in influencing the homeostasis of magnesium and manganese ions for the cure or control of human and bacterial infectious diseases. The results presented here unequivocally show that CalC protein is essential for the production of calcimycin, which is essentially a derivative of cezomycin, and allow us to propose a biosynthetic mechanism for calcimycin's production.

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Address correspondence to Zixin Deng, zxdeng@sjtu.edu.cn, or Zhijun Wang, wangzhijun@sjtu.edu.cn.

H.W. and J.L. contributed equally to this work.

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KEYWORDS calcimycin biosynthesis, cezomycin, oligomer, substrate-CoA ligase

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Calcimycin, a secondary metabolite produced by *Streptomyces chartreusis*, possesses a range of biological activities (1) and potential applications. As a molecule, it binds and transports divalent cations, including calcium, manganese, magnesium, and iron ions (1), and is capable of inhibiting the growth of Gram-positive bacteria and some fungi (2). It has also been observed to have a reducing effect on the metastatic potential of human colon cancer cells and to inhibit ATPase activity in mammalian cells as well as inducing cell death via direct activation of intracellular signaling processes linked to apoptosis (3–5). Calcimycin has also been used as a calcium transporter in experiments to promote the understanding of calcium signaling in human conditions such as heart disease (6), high blood pressure (7, 8), and brain disease (9–11). Further study of calcimycin and this class of compound will improve our ability to produce, manipulate, and apply the molecules in such a way that they can be rendered useful as commercial and medical products (12–15).

The sequence of the references is correct

N-demethyl calcimycin and cezomycin, the other two main polyether ionophores, consist of the same α-ketopyrrole, substituted benzoxazole, and spiroketal ring structure as those seen in calcimycin and differ only in their side group substitutions. See Fig. 1A for a representation of the molecules' structures. These compounds also accumulate in *S. chartreusis* NRRL 3882 (16).

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Our work has previously and partially confirmed the calcimycin biosynthetic pathway (16), in which CalN1 to CalN3, CalA1 to CalA5, and CalB1 to CalB4 proteins are responsible for the biosynthesis of the molecule's pyrrole, spiroketal polyketide ring, and benzoxazole moieties, respectively, and the CalR1 to CalR3 proteins are transcriptional regulators (16). The *calT* gene was observed to encode an integral membrane protein with significant sequence similarity to those of mycobacterial membrane protein large (MMPL) transporters and has been predicted to encode an antibiotic resistance protein (16). Previously, we have also observed that a *calB1* mutant accumulated compound 3 (Fig. 1A), whose structure possessed a full-length spiroketal polyketide ring and pyrrole moiety (17). Feeding that mutant with compounds structurally similar to 3-hydroxy anthranilic acid (3HA) permitted the formation of at least four additional new pyrrole spiroketal derivatives (17). CalM is an *S*-adenosylmethionine (SAM)-specific *N*-methyltransferase, catalyzing the N-methylation of the benzoxazole moiety (18). Tailoring steps in calcimycin biosynthesis include hydroxylation, amination at C-3, and N-methylation of the benzoxazole moiety (16). Five genes (*calC*, *calD*, *calF*, *calG*, and *calU3*) display extensive end-to-end identities with other proteins in the sequence database (16), but their roles in calcimycin biosynthesis are so far unknown and no biological function has yet been clearly assigned to the homologues of the CalU1, CalU2, CalU4, and CalU5 proteins.

From the above findings, it can be concluded that the biosynthetic relationship between calcimycin, N-demethyl calcimycin, and cezomycin was unclear, with one possibility being that 3HA may be modified to 6-amino-3HA and subsequently combined with the polyketide ring to generate calcimycin (Fig. 1B-I). Alternatively, 3HA might be combined with the polyketide ring first to generate cezomycin, which would then be modified to calcimycin (Fig. 1B-II).

Here we provide evidence supporting the latter hypothesis, i.e., that in *S. chartreusis* NRRL 3882 cezomycin is modified to produce calcimycin. Specifically, we report the identification and characterization of a new gene, *calC*, and its protein product, a coenzyme A (CoA) ligase, involved in the conversion.

RESULTS

Cezomycin is the modification precursor of calcimycin. We had hypothesized that a possible mechanism by which calcimycin was biosynthesized was that cezomycin was its precursor and was modified to form it. In initial experiments to test this hypothesis, we used the *calB1* mutant strain, which lacks the ability to produce

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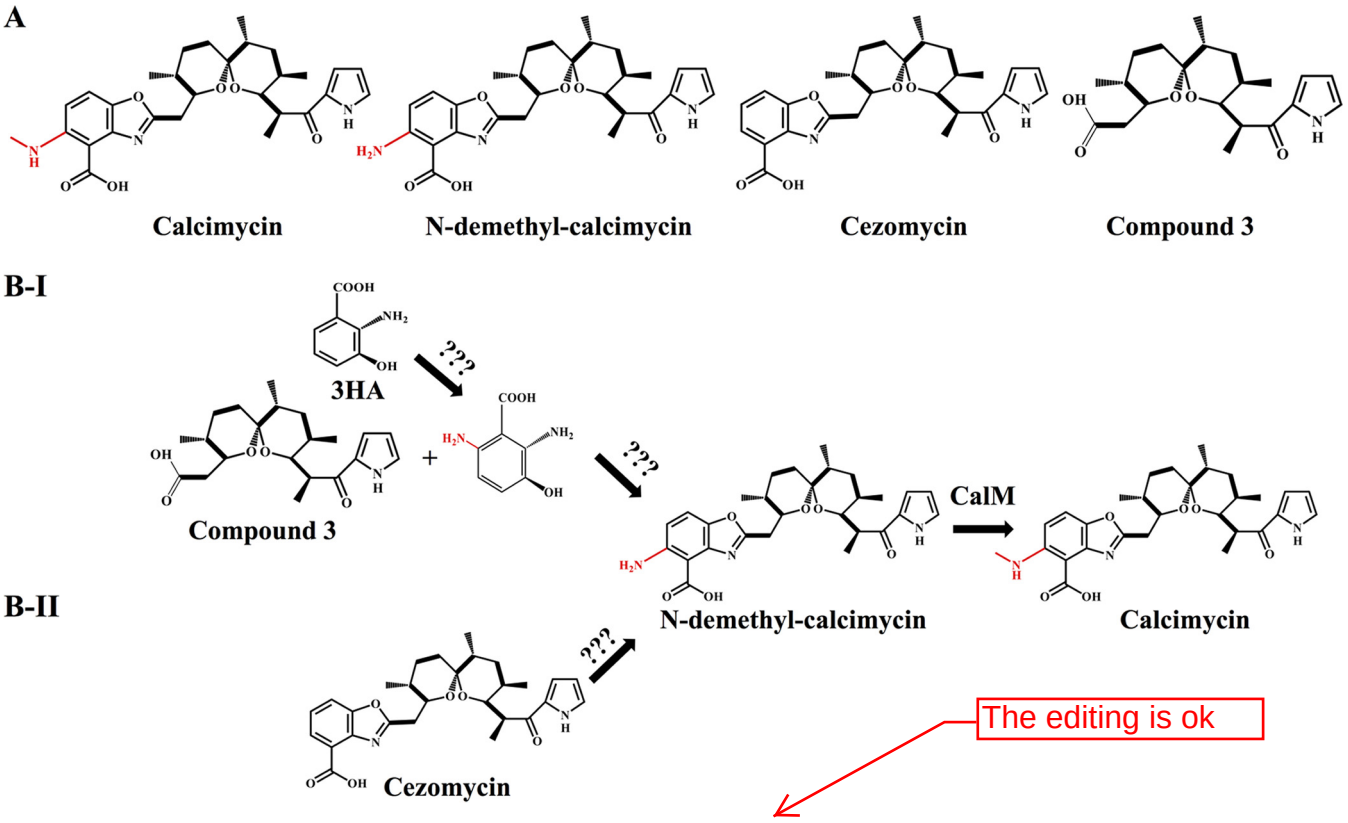

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FIG 1 Potential pathway(s) leading to the generation of calcimycin and its related compounds in *S. chartreusis* strains. Calcimycin, cezomycin, and N-demethyl calcimycin accumulate in *S. chartreusis* wild-type strain NRRL 3882. (A) Compound 3 accumulates in a *calB1* disruption mutant. (B-I, B-II) One possibility for the involvement of CalC, CalD, CalU3, and CalF in the step tailoring cezomycin to calcimycin consists of activation and modification of 3-hydroxyanthranilic acid, which will then combine with the polyketide spiroketal ring. The final release product could be either cezomycin or calcimycin, depending on whether 3-hydroxyanthranilic acid is modified (B-I). Alternatively, cezomycin could be the final release polyketide extension product, which is then modified by CalC, CalD, CalU3, CalF, and possibly other proteins to generate calcimycin (B-II). The key difference between the two possibilities is that in the former model, the generation of both cezomycin and calcimycin depends on the activation of 3-hydroxyanthranilic acid or its modification derivative, while in the latter model it does not. However, if calcimycin is generated through route B-I, then feeding cezomycin to the Δ*calB1* mutant strain should not have resulted in the production of calcimycin since the biosynthesis of the benzoxazole moiety is via 3-hydroxyanthranilic acid, whose production is blocked in the mutant.

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3-hydroxyanthranilic acid and accumulates compound 3 (Fig. 1A). 3-Hydroxyanthranilic acid is a precursor in the formation of the benzoxazole moiety (17), which in the wild-type calcimycin-producing strain is incorporated into both cezomycin and calcimycin (Fig. 1B-I and B-II). Consequently, the *calB1* mutant lacks production of both cezomycin and calcimycin. When the *calB1* mutant was fed with cezomycin, we

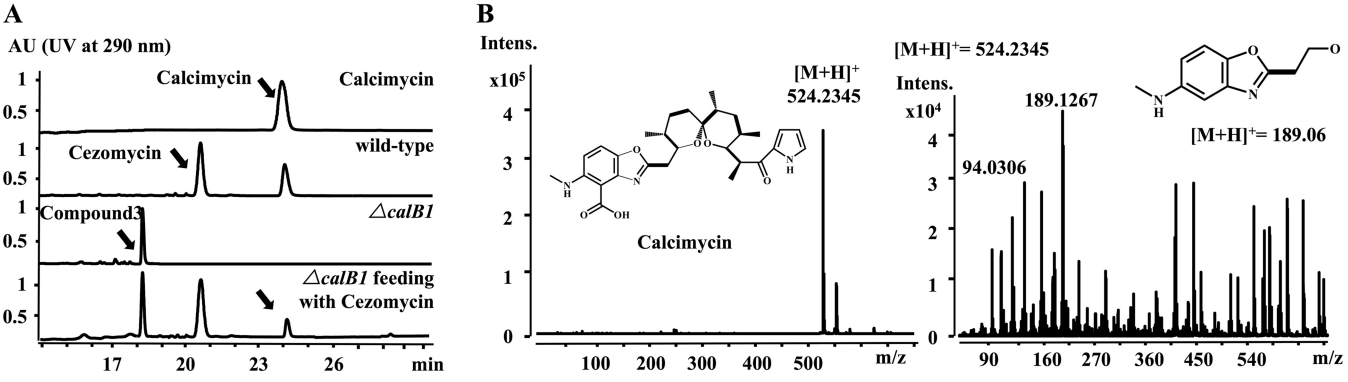

FIG 2 Restoration of calcimycin production by feeding the *calB1* deletion mutant (Δ*calB1*) with cezomycin. (A) The *calB1* mutant was cultured in SFM medium supplied with 0.02 mmol cezomycin. HPLC peaks of calcimycin, cezomycin, and compound 3 are marked. (B) Q-TOF analysis of product calcimycin identified in panel A. Labeled peaks are the characteristic mass fragmentation of *m/z* 94 ($C_5H_4NO_2$) of the pyrrole and *m/z* 189 of the benzoxazole moieties.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Streptomyces chartreusis strains		
NRRL 3882	Calcimycin production, wild type	NRRL
GLX4 (Δ*calC*)	*calC* deletion mutant, no calcimycin production	This work
GLX5 (Δ*calC/calC*)	Δ*calC* complementation strain, restores calcimycin production	
GLX6 (Δ*calD*)	*calD* deletion mutant	This work
GLX7 (Δ*calD/calD*)	Δ*calD* complementation strain	
GLX11 (Δ*calU3*)	*calU3* deletion mutant, no calcimycin production	This work
GLX12 (Δ*calU3/calU3*)	Δ*calU3* complementation strain, restores calcimycin production	
GLX18 (Δ*calF*)	*calF* deletion mutant	This work
GLX19 (Δ*calF/calF*)	Δ*calF* complementation strain	
Escherichia coli strains		
DH10B	*recA lacZ*ΔM15	Invitrogen
ET12567(pUZ8002)	Cml, Kan, *dam dcm hsdS* Tra^+ Cml	10
BW25113/pIJ790	RepA101(ts), *araBp-gam-be-exo*, AraC, RepA101(ts) Cml	11
BL21(DE3)/pLysS	F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal* λ(DE3) [pLysS Cml]	Stratagene
Plasmids		
p14F11	Cml	6
p6F5	Cml	6
pIJ773	Kan	11
pJTU2170	Integrative vector for gene complementation, *aac(3)IV* from pIB139 was replaced by *bla* and *neo* cassette	12
pET28a(+)	Plasmid for gene expression	Novagen
pET44b(+)	Plasmid for gene expression	Novagen
pJTU3662	pET28a(+)-derived plasmid for *calC* expression	This work
pJTU3663	pET28a(+)-derived plasmid for *calC* expression with ATP consensus domain deletion	This work
pJTU3664	pET44b(+)-derived plasmid for *calU3* expression	This work
pJTU3665	pET28a(+)-derived plasmid for *calF* expression	This work
pJTU3763	p14F11-derived plasmid carrying an apramycin resistance gene and a defective *calC*	This work
pJTU3764	p14F11-derived plasmid carrying an apramycin resistance gene and a defective *calD*	This work
pJTU3770	p6F5-derived plasmid carrying an apramycin resistance gene and a defective *calU3*	This work
pJTU3771	p6F5-derived plasmid carrying an apramycin resistance gene and a defective *calF*	This work
pJTU3777	pJTU2170-derived plasmid carrying *calC* for expression in *Streptomyces*	This work
pJTU3778	pJTU2170-derived plasmid carrying *calD* for expression in *Streptomyces*	This work
pJTU3780	pJTU2170-derived plasmid carrying *calU3* for expression in *Streptomyces*	This work
pJTU3784	pJTU2170-derived plasmid carrying *calF* for expression in *Streptomyces*	This work

[a]Cml, chloramphenicol resistance; Kan, kanamycin resistance; *aac(3)IV*, apramycin resistance.

the references in table 1 is wrong. 10 should be changed to 30, 11 to 31, 6 to 16, and 12 to 32

observed that its production of calcimycin was restored (Fig. 2A). As a double confirmation of the identity of the product, calcimycin, it was recovered by high-pressure liquid chromatography (HPLC) from the reaction mixtures and subjected to HPLC and high-resolution mass spectrometry. This unequivocally confirmed its identity (Fig. 2B).

The editing is ok

Identification and confirmation of the roles of the genes involved in cezomycin modification. Elucidation of the biological significance of the *calC*-, *calD*-, *calU3*-, and *calF*-encoded proteins in calcimycin biosynthesis was facilitated by (i) creation of related defective (calcimycin-nonproducing) mutants (Table 1) and (ii) their complementation with plasmids bearing the corresponding active gene under the control of a constitutive *ermE* promoter (Table 1). In the *calC*, *calD*, *calU3*, and *calF* mutant strains, the *apr* resistance gene had replaced most of the corresponding protein-coding sequence. Results from these experiments are shown in Fig. 3; see also Fig. S2 in the supplemental material. These data show that the *calC*, *calU3*, and *calF* genes are clearly involved in the modification of cezomycin (Fig. 3 and S2), as restoration/complementation of lost gene function restored product formation. The results for *calD* are less clear in that context.

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Biochemical characteristics of CalC protein. From its gene sequence, it is possible to deduce that CalC is an adenylate-forming enzyme that might activate the carboxylic acids for the subsequent biochemical biosynthesis (16) and that it is composed of 521

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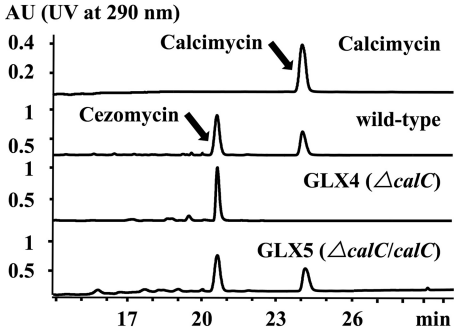

FIG 3 Phenotypic analysis of *calC* gene involved in the cezomycin modification pathway. HPLC analysis of calcimycin and cezomycin production in wild-type, *calC* mutant, and *calC* complementation strains.

AQ: D amino acids and is predicted to have a molecular mass of 57.2 kDa and a pI value of 8.26. The apparent molecular mass of the recombinant CalC-His protein in solution was F4 observed to be approximately 600 kDa, 10 times larger (Fig. 4B). This is likely to indicate that CalC protein adopts an oligomeric form in solution and is somewhat confirmed from the results of electron microscopy on CalC-His protein purified to homogeneity by Ni-affinity column and cation exchange and size exclusion chromatography (Fig. 4A, B, and C). In Fig. 4D (a typical image), particles with regular shapes can be observed. The mutant CalC protein lacking the entire ATP-binding region was subjected to a similar analysis and was also observed to display an oligomeric form similar to that of the functional CalC protein (see Fig. S3 in the supplemental material).

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CalC is an ATP-dependent cezomycin-CoA ligase. Pyrophosphate assay and product analysis with Q-TOF indicated that CalC possessed ATP hydrolysis activity. In these experiments, a CalC mutant in which the entire putative ATP-binding region was

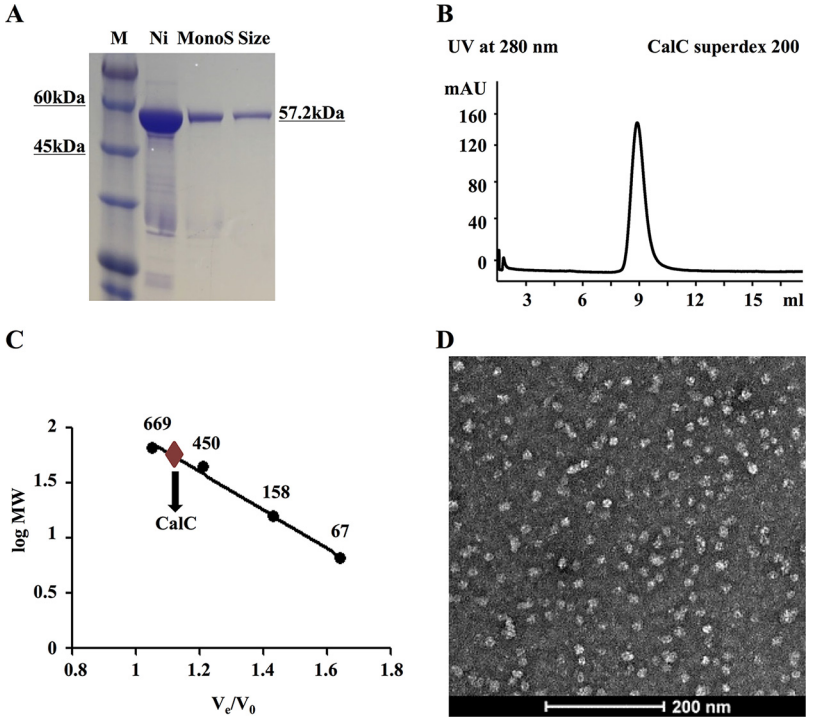

FIG 4 Characterization of CalC. (A) SDS-PAGE of CalC after purification using Ni, MonoS, and size exclusion columns. M, protein marker. (B and C) Thyroglobulin, ferritin, aldolase, and BSA were used as molecular mass markers for the estimation of the apparent molecular mass of CalC. (D) Potential oligomeric structure of CalC protein in solution as revealed by negatively stained electron microscopy (micrographic image).

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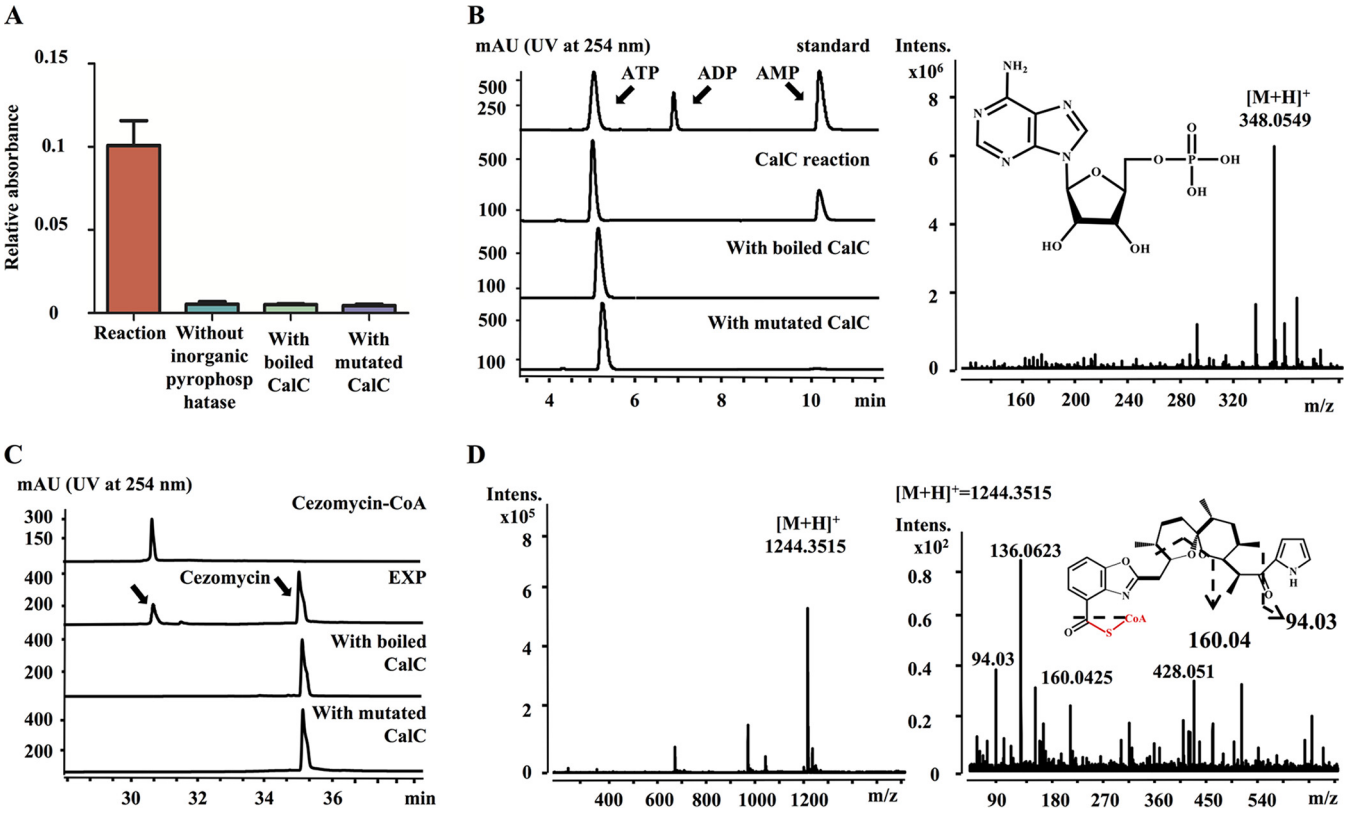

FIG 5 Catalytic activities of CalC protein. (A) ATP pyrophosphatase activity detection of CalC. A boiled sample (denatured) and the ATP catalytic domain deletion mutant of CalC were used as controls. (B) HPLC analysis of ATP hydrolysis product. Q-TOF analysis of AMP produced in the CalC-catalyzed reaction is also shown. (C) HPLC analysis of the reaction products catalyzed by CalC protein. (Cezomycin and CoA were detected by using Q-TOF LC-MS after the sample was digested in 0.1% KOH [Fig. S7]). (D) High-resolution mass spectrometry analysis of cezomycin-CoA. The mass fragment peaks of the pyrrole moiety (m/z = 94) and of benzoxazole (m/z = 160) of cezomycin are marked.

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deleted was used as a negative control, and the results showed that AMP is the ATP hydrolysis product of the reaction catalyzed by CalC and that CalC possesses ATP pyrophosphatase activity rather than ATPase activity (Fig. 5A and B).

In the presence of ATP, CoA-SH, $MgCl_2$, and cezomycin, it was the purified CalC-His protein, but not the heat-inactivated or ATP-binding mutant CalC protein, that catalyzed the production of cezomycin-CoA (Fig. 5C). Product confirmation was by reverse-phase HPLC/MS and its molecular mass/charge ratio (m/z), 1244.3515, was determined using high-resolution mass spectrometry and MS fragmentation peaks. This coincided well with those produced by a cezomycin-CoA standard used for comparison (Fig. 5D; see also Fig. S4 in the supplemental material). Compound 3, N-demethyl-calcimycin, calcimycin, the benzoxazole moiety precursor-3HA, and benzoate were not observed to be acted upon by the CalC protein (see Table S2 and Fig. S5 and S6 in the supplemental material). It is possible to suggest that the CalC protein is responsible for activating cezomycin to its CoA thioester adduct.

CoA-SH is coenzyme A (CoA), if the statement is misleading, it could be reworded to CoA

The activity of CalC was observed to be dependent upon the presence of either magnesium or manganese ions but not of calcium or iron ions (see Fig. S7 in the supplemental material).

CalC kinetic properties. The apparent K_m values for cezomycin, ATP, and CoA were measured as 190 ± 25, 200 ± 32, and 485 ± 67 μM, respectively, at 30°C. The corresponding k_{cat} values for cezomycin, ATP, and CoA were determined to be 3.98 ± 0.12, 4.00 ± 0.15, and 4.21 ± 0.17 min^{-1}, respectively (Table 2; see also Fig. S8 in the supplemental material). However, in the absence of inorganic pyrophosphatase, the apparent K_m values for cezomycin, ATP, and CoA were measured as 219 ± 22, 233 ± 25, and 658 ± 96 μM, respectively. The corresponding k_{cat} values for cezomycin, ATP,

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TABLE 2 Apparent kinetic values for cezomycin, CoA, and ATP

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Cezomycin	190 ± 25	3.98 ± 0.12	349
CoA	485 ± 67	4.21 ± 0.17	144
ATP	200 ± 32	4.00 ± 0.15	333

and CoA were determined to be 1.60 ± 0.04, 1.61 ± 0.04, and 1.51 ± 0.05 min^{-1}, respectively (see Table S3 in the supplemental material).

***In silico* analysis of the proteins proposed to be involved in calcimycin biosynthesis. (i) CalC protein.** *In silico* protein sequence analysis of CalC indicated that it possessed similarity with known acyl-CoA ligases, demonstrating a high level of sequence identity with the acyl-CoA ligase CreM of *S. cremeus* (44%) and lower levels of sequence identities with three other acyl-CoA ligases, namely, SanJ of *Streptomyces ansochromogenes* (21%), VisB of *Streptomyces virginiae* (18%) and SdgA of *Streptomyces* sp. strain WA46 (19%) (see Fig. S9 in the supplemental material). All those other acyl-CoA ligases and CalC possess an adenylation domain with conserved sequences that appear in a defined order (19–23). The adenylation domain is thought to be responsible for the enzyme's carboxyl substrate specificity and is responsible for the generation of the substrates corresponding to carboxyl adenylate adduct in the presence of ATP (24).

These observations confirm and support the hypothesis that we have presented above based upon results from our experiments, i.e., that CalC functions as a CoA ligase in the synthesis of calcimycin from cezomycin in the presence of ATP.

(ii) CalD protein. Our previous *in silico* analysis of CalD protein indicated that it resembled NAD(P)H-dependent oxidoreductases/dehydrogenases (16), and results from experiments reported here have shown that its physical disruption resulted in a slight decrease, but not abolishment, of the production of calcimycin. Consequently, the exact function of the gene in calcimycin biosynthesis is, unfortunately, still unclear.

***calU3* and *calF* genes.** From our previous *in silico* analysis of the *calU3* and *calF* (16) genes, we know that they share significant DNA sequence identities with the *creE* (68%) and *creD* (60%) genes of *Streptomyces cremeus* (25), respectively. The CreE and CreD proteins are known to be responsible for the formation of nitrous acid, which has a role in diazo group generation in this organism's biosynthesis of the ortho-diazoquinone containing secondary metabolite cremeomycin (25). In this work, we report that the disruption of *calU3* and *calF* genes diminished the production of calcimycin significantly (Fig. S2) but that the addition of inorganic nitrite ($NaNO_2$) to the culture medium of such mutants restored their production of calcimycin (see Fig. S10 in the supplemental material). Further, our *in vitro* study revealed the involvement of CalU3 and CalF in generating nitrous acid (see Fig. S11 in the supplemental material).

Put together, these observations suggest that the *calU3* and *calF* genes are involved in specifying proteins involved in the biosynthesis of calcimycin from cezomycin by way of the formation of nitrous acid.

DISCUSSION

Our results reported here support the view that calcimycin is derived from cezomycin in a reaction catalyzed by the CalC protein that is energy dependent, transforming ATP to AMP; CalC functions as a CoA ligase and catalyzes the conversion of cezomycin to cezomycin-CoA. Our data might also suggest that cezomycin might then be further modified to calcimycin (Fig. 6). It was suspected that in the calcimycin production there might be a hydroxylation step at the C-3 position of cezomycin and the CalD protein might be responsible for this step (16). The CalD protein was implicated previously as an orthologue to known NAD(P)H-dependent oxidoreductases (16), and yet the deletion of the gene did not obviously affect calcimycin/cezomycin metabolism. Therefore, whether there is such a hydroxylation step of cezomycin involving CalD remains unclear. Since our evidence revealed that supplement of

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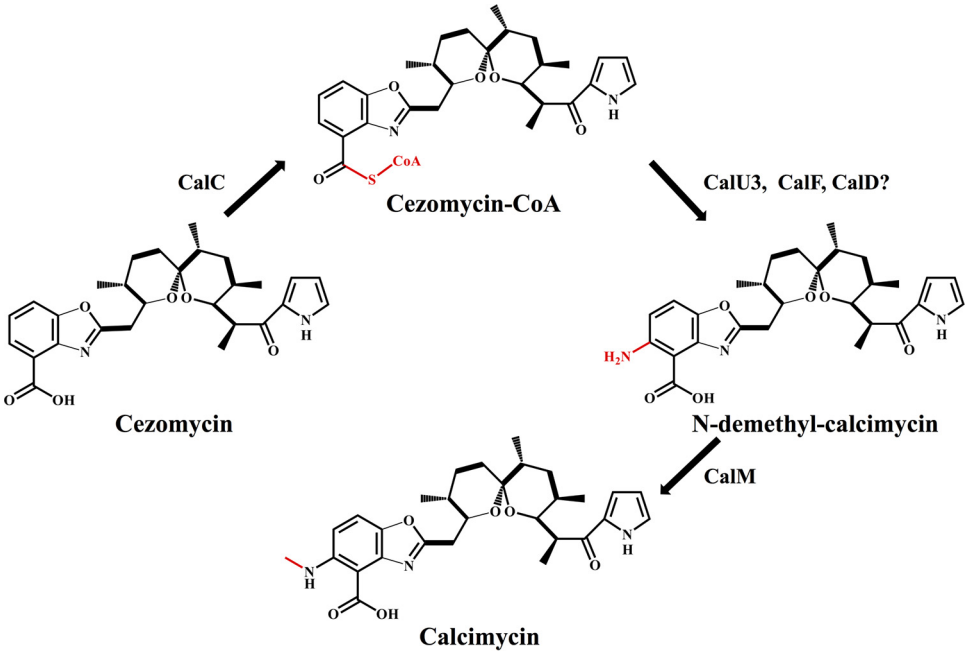

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FIG 6 A possible cezomycin modification pathway in calcimycin biosynthesis. Cezomycin is converted to cezomycin-CoA by CalC. The compound is then possibly modified by CalD, CalU3, or CalF (and perhaps other proteins) to generate N-demethyl-calcimycin with a final methylation by CalM to yield calcimycin.

inorganic nitrite ($NaNO_2$) to *calU3* and *calF* mutant strains restored their calcimycin production and the CalU3 and CalF proteins can generate nitrous acid (Fig. S2, S10, and S11), it is therefore reasonable to suggest that the two genes are involved in providing the nitrogen source for the amination of cezomycin at its C-3 position (16).

The biosynthetic mechanism suggested above, i.e., the modification of a closely related calcimycin precursor (which possesses no or significantly reduced biological activity), might allow a significant protective advantage for the producing host bacteria. In that context, although ionophore-mediated transport of specific ions across cell membranes has been well studied (14, 26, 27), little is known about the physiological effects of the molecules on their bacterial producing organisms (28). The results of our work allow us propose the possibility that calcimycin (polyether divalent cation ionophore)-producing organisms can avoid the likely negative consequences of the intracellular presence of such molecules (possible cation depletion) by "stockpiling" a close precursor, cezomycin, that possesses a binding affinity for cations 10 times less than that of calcimycin (1). In times of need (metabolic stress or environmental competition from other organisms [28]), such a precursor could then be rapidly converted to its "active form," in this case, calcimycin. In this context, the K_m of CalC for cezomycin is around 190 μM, and this concentration may be of physiological relevance.

MATERIALS AND METHODS

Bacterial strains, genomic DNA, plasmids, and culture conditions. Bacterial strains and plasmids used in the study are listed in Table 1. *Escherichia coli* plasmid isolation, gene cloning, and other routine molecular biological procedures were performed as described by Sambrook and Russell (29). *S. chartreusis* NRRL 3882 genomic DNA was isolated according to the protocol of Kieser et al. (30).

Escherichia coli strains were maintained and grown in or on liquid or solid Luria broth (LB). Small-scale growth of *S. chartreusis* NRRL 3882 and its derivative strains was by culture in TSBY liquid medium, containing 3% tryptone soy broth, 10.3% sucrose, and 0.5% yeast extract (for extraction of chromosomal DNA), or on SFM agar, containing 2% mannitol, 2% soybean powder, and 2% agar (pH 7.2) (for sporulation and conjugation). Liquid fermentation of *S. chartreusis* NRRL 3882 and its derivative mutant strains was performed in SFM medium without agar. Media were supplemented when necessary with 50 μg liter^{-1} apramycin.

Inactivation and complementation of *calC*, *calD*, *calU3*, and *calF* genes. The *calC*, *calD*, *calU3*, and *calF* genes in *S. chartreusis* strain NRRL 3882 were replaced by the *apr* resistance gene, using Redirect Technology (31) as described in the product literature. Briefly, the *apr* resistance gene from pIJ773 (31)

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TABLE 3 Primers used in this study

Primer	Sequence (5′–3′)[a]	Use
C-F1	CCTGGGCGAGCAGGACCGTTACGACCAGCAGGTCACCGA**ATTCCGGGGATCCGTCGACC**	Replacement of *calC* by Redirect Technology
C-F2	TCAGGCGGCCCGTTCCGCGAGGAGACGGGCCAGCTCCAG**TGTAGGCTGGAGCTGCTTC**	
C-F3	GCGAGTCCACGTGGACCATG	PCR analysis of GLX4 (Δ*calC*)
C-F4	AGCACCTTCGACGCCATCCA	
comC-F1	CCGGAATTCCACCTGGAACGAGAAGATCCC	PCR amplification of *calC* for complementation
comC-F2	GGAATTCCATATGATTCTGCAACGCATAGCG	
28awhC-F1	GGAATTCCATATGATTCTGCAACGCATAGCGAAC	Amplification of *calC* for expression
28awhC-F2	CCGCTCGAGTCAGGCGGCCCGTTCCGCGAG	
28awhC-F3	CGCACACGCCCAAGCTGGCCGTGCACACCGGCCGCACCC	Amplification of *calC* for expression with ATP consensus domain deletion
28awhC-F4	GGCGTGTGCGTGATCAGTGTGGGGTGGTCCGGTGGCATG	
D-F1	TTCCGCACGCCCATGGACTTCCCGTTCGTCATCAGCCGCATTCCGGGGATCCGTCGACC	Replacement of *calD* by Redirect Technology
D-F2	GTTGATGACGTCGGCCGCTTCGGCCAGCTCCGTCACCCGTGTAGGCTGGAGCTGCTTC	
D-F3	ATGCAGGCAGCCTTCATCGA	PCR analysis of GLX6 (Δ*calD*)
D-F4	CTATCGCAGGGCCCCGGCCC	
comD-F1	CCGGAATTCCTATCGCAGGGCCCCGGCCC	PCR amplification of *calD* for complementation
comD-F2	GGAATTCCATATGAGGCAGCCTTCATCGAGCG	
U3-F1	CCGCGTCAGGAGCGCACCGCGACCCTGGCCCGCATCCAC**ATTCCGGGGATCCGTCGACC**	Replacement of *calU3* by Redirect Technology
U3-F2	CCGGTGCGAGTTGCCCTCCAGACCCCCGTGGTCCACGGC**TGTAGGCTGGAGCTGCTTC**	
U3-F3	CGGTTGAACAGTCTGGACGC	PCR analysis of GLX11 (Δ*calU3*)
U3-F4	TCCGCGATCCGGGAGGCCGG	
comU3-F1	CCGGAATTCTCACACGATCACCCCGGTCA	PCR amplification of *calU3* for complementation
comU3-F2	GGAATTCCATATGAACGGCACCATGGAGATCTG	
44bU3-F1	GAATTCCATATGAACGGCACCATGGAGATCTGC	Amplification of *calU3* with Strep Tag II at C terminus for expression
44bU3-F2	CTTCCTCGAGTCACTTTTCGAACTGCGGGTGGCTCCACACGATCACCCCGGTCAGGTC	
F-F1	TGCCGGACGCTGCGCGTCATCCGGGGCGACCTGGCGCGG**ATTCCGGGGATCCGTCGACC**	Replacement of *calF* by Redirect Technology
F-F2	GCCCGTCAGCCGCAGGCACTCCCGCAGCAACTGCCACTC**TGTAGGCTGGAGCTGCTTC**	
F-F3	GCCAACCCGGTGGTCGGTGT	PCR analysis of GLX18 (Δ*calF*)
F-F4	TCCGGCCGGACCTCCAGCCC	
comF-F1	CCGGAATTCCTACCGGGCCAGTGCCCGGT	PCR amplification of *calF* for complementation
comF-F2	GGAATTCCATATGCTGGACGCCGAGGCAGCGTT	
28aF-F1	GGATCCATATGCTGGACGCCGAGGCAGCG	Amplification of *calF* for expression
28aF-F2	CCGCTCGAGCTACCGGGCCAGTGCCCGGTCCAC	

[a]Boldface indicates ●●●.

the boldface indicates the **20 and 19 nt sequences for needed the amplification of apramycin resistance gene**

(Table 1) was amplified using KOD-plus DNA polymerase (Toyobo Biotech Co. Ltd.) and *calC*, *calD*, *calU3*, and *calF* gene-specific primers (Table 3). The resulting amplification products were introduced into *E. coli* BW25113/pIJ790 harboring p14F11 or p6F5 (16) to generate plasmids pJTU3763 (Δ*calC*), 3764 (Δ*calD*), pJTU3770 (Δ*calU3*), and pJTU3771 (Δ*calF*). These plasmids were then introduced into *S. chartreusis* NRRL 3882 by conjugation with *E. coli* ET12567/pUZ8002, and double crossover Apr^r mutants were isolated by selection on SFM medium containing apramycin. The identity of the mutants was confirmed by PCR and double-stranded sequencing of amplification products.

Gene complementations of *S. chartreusis* Δ*cal* mutant strains were achieved by introducing plasmids possessing full-length complementary DNA into the gene deletion strains. Briefly, *calC*, *calD*, *calU3*, or *calF* genes were PCR amplified from the purified genomic DNA of *S. chartreusis* NRRL 3882 strain by using gene-specific primers (Table 3). After size and sequence confirmation, PCR products were ligated into vector plasmid pJTU2170 (32) to generate complementation plasmids, namely, pJTU3777 (*calC*), pJTU3778 (*calD*), pJTU3780 (*calU3*), and pJTU3784 (*calF*), and stabilized *in E. coli* ET12567. These plasmids were then introduced into *S. chartreusis* Δ*cal* strains GLX4 (Δ*calC*), GLX6 (Δ*calD*), GLX11 (Δ*calU3*), or GLX18 (Δ*calF*) (Table 1) via conjugation with the appropriate *E. coli* ET12567 complementation-bearing strain to produce GLX5 (Δ*calC*/*calC*), GLX7 (Δ*calD*/*calD*), GLX12 (Δ*calU3*/*calU3*), or GLX19 (Δ*calF*/*calF*). Gene expression in the complementation plasmids was constitutive and under the control of an *ermE* promoter (30). Complemented conjugation products were selected by virtue of their kanamycin resistance (growth on LB supplemented with 50 μg liter^{-1} kanamycin), and their identities were confirmed by PCR amplification and sequencing of the full-length gene.

LC/MS analysis of molecules of interest from fermentation culture. For liquid chromatography-mass spectrometry (LC/MS) analysis, *S. chartreusis* NRRL 3882 (wild type) and its derivative Δ*calC*, Δ*calD*, Δ*calU3*, Δ*calB1* (17), and Δ*calF* mutant strains were precultured in 10 ml of TSBY medium in a 50-ml conical flask at 30°C with gentle shaking at 220 rpm for 48 h, after which 5 ml of the resultant cultures was aseptically removed and inoculated into 500-ml baffled flasks containing 100 ml liquid SFM medium (pH 7.3). Cultivation was continued at 30°C with shaking at 220 rpm for 9 days. For the determination of strain GLX Δ*calB1*'s ability to convert cezomycin to calcimycin, cezomycin feeding experiments were conducted, in which 0.02 mmol of cezomycin dissolved in 0.5 ml dimethyl sulfoxide (DMSO) was added to the culture 2 days following inoculation of the mutant spores in liquid SFM medium, and the culture was allowed to incubate for a further 7 days. After this time, the 100 ml was centrifuged at 6,000 × *g* for 30 min to remove cells and cellular debris, and the supernatant was used to assay for calcimycin after extraction with 1.5 volumes of ethyl acetate. Extracted calcimycin was dried under vacuum in a rotary

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evaporator and then redissolved in 0.5 ml methanol and assayed using an Agilent 1100 series LC/MSD Trap system (Agilent Technologies, Tokyo, Japan) fitted with an Agilent Zorbax SB-C18 (4.6- by 150-mm) column (Agilent Technologies). Sample separation was performed using gradient mixtures of solution A (0.1% formic acid in water) and solution B (0.1% formic acid in methanol), as follows: 75% to 85% of solution B for 8 min, followed by 85% to 95% for 14 min, 95% to 100% for 7 min, and finally 100% of solution B for 6 min at a flow rate of 0.4 ml min^{-1}. Eluate was monitored by UV adsorption at a wavelength of 280 nm ($\lambda_{280\ nm}$), and a calcimycin standard was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) for comparison. Eluate producing a peak at $\lambda_{280\ nm}$ was collected and subjected to high-resolution mass spectrometry using an Agilent 6530 Series Accurate-Mass quadrupole time of flight (Q-TOF) LC/MS (Agilent Technologies) to reconfirm product identity by M_r.

Synthesis and purification of cezomycin and cezomycin-CoA. *S. chartreusis* NRRL 3882 was cultured for 9 days in 15 1-liter conical flasks, each containing 400 ml SFM medium, to make 6 liters at 30°C while gently shaking at 220 rpm, after which cells and cellular debris were removed by centrifugation at 6,000 × *g* for 30 min in 500-ml polypropylene centrifuge bottles in an Eppendorf 5810 R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was then divided into 500-ml volumes, each of which was carefully transferred to a 2-liter separating funnel and thoroughly mixed with 750 ml of dried ethyl acetate. The mixture was allowed to settle for 10 min to permit phase separation, and the upper ethyl acetate layer was collected, placed in a rotary evaporator, and dried *in vacuo* for 1 h. The dried product (from 6 liters of fermentation broth) was redissolved in 5 ml 90% (vol/vol) aqueous methanol, layered onto a reversed-phase silica gel (AAG 12S50; YMC Co. Ltd.) column, and eluted with aqueous methanol in a gradient from 70% to 100% at a flow rate of 0.5 ml min^{-1}. Ten-milliliter fractions were collected from the column, and 20 μl from each fraction was analyzed using HPLC/MS as described above. Cezomycin identity in the eluate was confirmed by comparison of HPLC retention time, UV spectrometry, and mass with a cezomycin standard (16). Fractions, around 20 ml, containing cezomycin were pooled and concentrated in a rotary evaporator under vacuum for 1 h. Typically, approximately 60 mg cezomycin can be obtained from 6 liters liquid culture.

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The *in vitro* synthesis of cezomycin-CoA was conducted using the method of Belshaw et al (33), and all necessary reagents were purchased from Sigma-Aldrich (Merck KGaA). Briefly, 10 mg cezomycin (0.02 mmol) was mixed with 30 mg coenzyme A (0.03 mmol), 25 mg (0.05 mmol) PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate), and 30 mg potassium carbonate (0.2 mmol) in a 50-ml conical flask, and 5 ml of a 1:1 THF (tetrahydrofuran)-water mixture was added. The solution was gently mixed for 2 h at room temperature, after which the precipitate was removed by centrifugation at 15,000 × *g* for 10 min. The supernatant containing the cezomycin-CoA adduct was loaded onto a YMC column (AAG 12S50; YMC Co. Ltd.) and eluted with a gradient of 10 mM ammonium acetate in aqueous methanol from 10% to 100% over a 240-ml volume. Five-milliliter fractions were collected, from which 20 μl was removed for the assay of cezomycin-CoA by HPLC/MS using an Agilent Zorbax SB-C_{18} (4.6- by 150-mm) column at a flow rate of 0.4 ml min^{-1}, and solution A (10 mM ammonium bicarbonate, pH 6.7) was gradually replaced by 2 to 30% volumes of solution B (methanol) over a period of 8 to 14 min, 30 to 70% volumes for 14 to 25 min, and then 70 to 100% volumes for 25 to 28 min. The elution was monitored by UV spectroscopy at $\lambda_{254\ nm}$. Fractions containing cezomycin-CoA (around 10 ml) were pooled and concentrated under vacuum in a rotary evaporator. The molecular identity of the cezomycin thioester derivative was confirmed by Q-TOF LC-MS (Agilent 6530 series accurate-mass Q-TOF LC/MS; Agilent Technologies) by an ion peak at *m/z* of 1,244.34 (see Table S1 and Fig. S1 in the supplemental material).

Cloning, expression, and purification of CalC-His protein. The *calC* gene was cloned in frame with the poly-histidine codons in the pET28a(+) expression vector (Novagen, Merck KGaA) according to the manufacturer's instructions. Initially, the *calC* gene was amplified by PCR using purified genomic DNA extracted from *S. chartreusis* NRRL 3882 and primers 28awhC-F1 and 28awhC-F2 (Table 3), and after product identity confirmation by agarose gel electrophoresis and bidirectional sequencing it was ligated into pET28a(+), which had been linearized by double digestion with NdeI and XhoI. The ligation product, pJTU3662, was stabilized by introduction into $CaCl_2$-treated *E. coli* DH10B (29).

Similarly, a *calC* gene mutant with an altered ATP binding domain was also cloned. Briefly, gene-specific primers 28AwhC-F3 and 28AwhC-F4 (Table 3) were used to PCR amplify a target DNA fragment from the cloned *calC* gene on plasmid pJTU3662. The resulting PCR product contains homologous recombinant arms, which facilitate its cyclization once introduced into host *E. coli*. Introduction of the PCR fragment into *E. coli* strain DH10B resulted in the generation of plasmid pJTU3663, which was reisolated, purified, and transformed into *E. coli* strain BL21(DE3)/plysS (Agilent Technologies) for use in heterologous gene expression.

Production of cloned poly-His-tagged *calC* and Δ*calC* mutant protein was done according to the protocols given by Novagen and was achieved by growth of $CalC^+$ and $CalC^-$ *E. coli* strains in 1 liter of LB medium at 37°C containing 50 μg ml^{-1} kanamycin and 25 μg ml^{-1} chloramphenicol with shaking at 250 rpm to an $A_{600\ nm}$ of 0.6. Protein expression was then induced by addition of 1 ml of 0.4 mM isopropyl-D-thiogalactopyranoside (IPTG) solution, and the culture was allowed to incubate for a further 24 h at 16°C. Cells were then harvested by centrifugation and stored frozen at −80°C for subsequent protein extraction and purification.

Recombinant His-tagged CalC and CalC ATP binding mutant proteins were recovered from 4 g of frozen *E. coli* cells that were thawed on ice and resuspended in 50 ml of buffer A (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% glycerol, 40 mM imidazole, and 1 mM ATP) and lysed by sonication. Cell debris was removed by centrifugation at 20,000 × *g* for 40 min at 4°C, and the resulting supernatant was loaded onto a nickel-nitrilotriacetic acid (NTA) resin, HisTrap HP 1-ml column (GE Healthcare Life Sciences, Little

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Chalfont, UK) preequilibrated with buffer A. The column was then washed with 5 bed volumes of buffer C (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% glycerol, and 80 mM imidazole) to facilitate His-tagged protein binding, followed by 2.5 bed volumes of elution buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5% glycerol, and 500 mM imidazole). The total eluate following elution buffer addition (2.5 ml) was collected and loaded onto a Mono S 5/50 GL column (GE Healthcare Life Sciences), preequilibrated with buffer D (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, and 5% glycerol), and eluted at a rate of 1 ml min^{-1} by addition of a linear gradient of NaCl at concentration of 50 mM to 1 M. Three-milliliter fractions were collected and monitored for CalC-His protein using SDS-PAGE. Ten microliters of each fraction was loaded onto a 15% polyacrylamide gel along with a size marker (Tiangen, Shanghai, China) for comparison. Samples were electrophoresed at a constant 25 V for 1 h or until the loading dye had reached the bottom of the gel. Fractions identified as containing protein were stored frozen at −80°C until required.

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Determination of the apparent molecular mass of the CalC-His protein. Peak fractions as described above identified as possessing CalC-His protein and its related ATP binding mutant protein were further purified on a Superdex 200 10/300 GL column (GE Heathcare Life Sciences) using an elution solution of 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 5% glycerol. The column was first size calibrated using four molecular mass markers (thyroglobulin, 669 kDa; ferritin, 450 kDa; aldolase, 158 kDa; bovine serum albumin [BSA], 67 kDa; obtained from GE Healthcare Life Sciences) (29) and the molecular mass of CalC-His protein was obtained by comparison in terms of its elution point (29).

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Electron microscopy. CalC-His protein and the related ATP binding mutant proteins were imaged on a Tecnai 12 transmission electron microscope (EM; Thermo Fisher Scientific, Waltham, MA, USA) operating at 120 keV and at a magnification of ×42,000 with a nominal defocus ranging from −0.8 to −0.3 μm. Images were acquired using a Gatan Eagle 4k × 4k charge-coupled-device (CCD) camera (Thermo Fisher Scientific), with a final pixel resolution size of 2.71 Å.

CalC and mutant protein activity assays. An EnzChek pyrophosphate assay kit (product number E-6645; Thermo Fisher Scientific) was used to assess the activities of CalC-His and CalC-His mutant protein using the method described in the manufacturer's literature. The activity assay buffer employed in these experiments contained 50 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM ATP, 0.5 mM CoA, and 0.2 mM cezomycin, and the total reaction volume was 100 μl. Reactions were initiated by addition of 0.05 mg of purified CalC-His or CalC-His mutant or boiled (denatured) protein (as a negative control) to the reaction mixture. Reactions were performed in triplicate in a quartz cuvette, and the absorbance of the solution at $\lambda_{360\ nm}$ was measured continuously at 30°C over a period of 2 h in a PerkinElmer Lambda 650 spectrophotometer (PerkinElmer, Waltham, MA, USA). This allowed the conversion of the substrate, 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG), to ribose 1-phosphate and 2-amino-6-merc apto-7-methylpurin to be monitored. The reaction rate was calculated by plotting the change in absorbance against time and measuring the slope of the graph.

Triplicate assays were also performed in microcentrifuge tubes at 30°C, in a similar manner but with 0.2 mM each of the following substrates: cezomycin, N-demethyl-calcimycin, calcimycin, 3HA, and benzoate. In this case, enzymatic reactions were terminated by protein precipitation by addition of 100 μl of methanol after 2 h. The precipitated protein was removed after centrifugation at 15,000 × *g*, and the supernatant was removed and analyzed using an Agilent 6530 Series Accurate-Mass Q-TOF LC/MS (Agilent technologies). In the various assays performed, $MgCl_2$ was sometimes replaced with $MnCl_2$, $FeCl_2$, or $CaCl_2$ to assess if these divalent cations could influence the reaction.

Initial reaction velocities of CalC-His proteins with cezomycin, CoA, or ATP were measured in triplicate by substrate concentration variation, one at a time, while keeping that of the other two saturated, which can be determined with several rounds of preliminary testing. The concentrations of CoA, cezomycin, and ATP were varied between 0.01 and 3 mM for determination of their apparent K_m values. In a separate series of experiments, it had been deduced that none of the three compounds showed any enzyme inhibition up to that value. A series of 100-μl reaction mixtures in microcentrifuge tubes containing 50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 5% glycerol, 2 μM CalC, and 1 mM $MgCl_2$ with or without 1 U inorganic pyrophosphatase were set up, the reactions were initiated by addition of CalC protein, and the mixtures were incubated at 30°C for 4 h, with assays performed starting 5 min after setup and at 15-min intervals. At each assay point, the entire contents of the tube (in triplicate) were assayed for cezomycin-CoA by HPLC/MS as described previously (Agilent 6530 series accurate-mass Q-TOF LC/MS). Cezomycin-CoA synthesized and purified in this project was used for standard curve construction, which was used for comparison with values generated from reaction samples. Prism5 software (Graph-Pad Software, Inc.) was used for the calculation of kinetic parameters.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00586-18.

SUPPLEMENTAL FILE1, PDF file, 1.3 MB.

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AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 1

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AQA—To ensure sequential order, references have been renumbered in the text and References (Materials and Methods has been moved to the end of the text per ASM style; also, former references 14 and 33 were duplicates). Please check and correct the renumbering if necessary. If any reference should be deleted from the References list, please mark "Reference deleted" in the margin next to that entry; do not renumber subsequent references.

AQB—If "performance" is better than "pressure" for the definition of HPLC, please correct.

AQC—In Table 1, strain names such as "GLX 5" have been changed to "GLX5" (with no space before the number) to match instances in the text and other tables and the companion manuscript. Okay to change "*kana*^{r}" to "Kan" for pIJ773? If not, please explain "*kana*^{r}."

AQD—M_r changed to "molecular mass" because a size in kDa is given (M_r is a unitless measure).

AUTHOR QUERIES

AUTHOR PLEASE ANSWER ALL QUERIES 2

AQE—Please define "CoA-SH."

AQF—Because Materials and Methods has been moved to the end of the text, Tables 2 and 3 were switched and renumbered as Tables 3 and 2, respectively. Please check renumbering throughout.

AQG—In Table 3 (formerly Table 2) footnote, please explain what boldface represents.

AQH—"in the previous section" has been changed to "as described above" in "20 μl from each fraction was analyzed using HPLC/MS as in the previous section." If you wish to be more specific, please give the exact wording of the heading of the section referred to.

AQI—"molecular weight" has been changed to "molecular mass" when units are given (molecular weight is a unitless measurement).

AQJ—If the sentence beginning with "One possibility for the involvement of CalC, CalD," in the legend to Fig. 1 is not okay as edited, please reword to clarify "...CalF cezomycin tailoring step to calcimycin..."