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Characterization of the Bafilomycin Biosynthetic Gene Cluster from *Streptomyces lohii*

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Keywords

bafilomycin; plecomacrolide; polyketide; biosynthesis; secondary metabolite

Bafilomycin B₁ is the archetypal compound of the plecomacrolide family of natural products, which include concanamycins, viranamycins, hygrolidin, elaiophyllin, and allied metabolites.¹ This compound was first isolated from *Streptomyces griseus* sp. sulphurus (TÜ 1922) in 1984.² The term plecomacrolide was later introduced to describe a subgroup of polyketides featured with a 16- or 18-membered macrolactone containing two conjugated diene units, and a hemiacetal side chain.¹ These compounds have now been studied extensively as vacuolar ATPase (V-ATPase) inhibitors and, as such, have shown some promise as anti-osteoporotics.^{3,4} In addition, synergistic antifungal activity of bafilomycin A₁ with the calcineurin inhibitor FK506 against the life-threatening fungal pathogen *Cryptococcus neoformans*,⁵ inhibition of the release of β -amyloid by bafilomycin A₁,⁶ together with antitumor,⁷ antiparasitic,⁸ and immunosuppressant⁹ activities of other bafilomycins were reported. Despite continued efforts, generalized toxicity has kept these compounds from clinical application. Semi-synthetic derivatives have provided some indication that target specificity can be enhanced.¹⁰ However, extensive manipulation of the core structure for structure-activity relationship studies remains hampered by the complexity of total synthesis.^{11,12} Decades of work in the field of polyketide biosynthesis have yielded tools to generate subtle changes to the core macrolide via genetic manipulation.^{13,14} In order to utilize this knowledge to generate novel analogues, the native biosynthetic gene cluster sequence must be elucidated. When a bafilomycin-producing organism, *Streptomyces lohii*, was isolated during an ongoing drug discovery program in our laboratories, the characterization of the bafilomycin biosynthetic pathway was initiated.

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Supporting information for this article is available

To isolate the genes involved in bafilomycin biosynthesis, a fosmid library was constructed from *S. lohii* (ATCC BAA-1276, JCM 14114) genomic DNA. This library was probed for type I polyketide ketosynthase (KS) domains using a radiolabelled PCR product amplified from *S. lohii* gDNA using degenerate primers. Colony hybridization experiments revealed 12 KS positive fosmids. Initial restriction digestions and Southern hybridization experiments revealed two major groups of overlapping fosmids. One clone from each group was chosen for further analysis. BLAST analysis¹⁵ of the sequence from a small number of subclones suggested that one of these representatives contained only type I polyketide synthase (PKS) genes. Complete sequencing of this clone revealed the domain organization of the PKS modules to exactly match with a section of the genetic architecture predicted by the structure of bafilomycin and the typical polyketide rules of colinearity. End sequencing of the other KS positive fosmids identified those containing overlapping sequence. These were also sequenced to generate a map of the entire gene cluster.

The macrolactone core of bafilomycin is assembled by a type I PKS system. These genes are arranged as five open reading frames, *bafAI*–*bafAV*, spanning 59 kilobases (Scheme 1, Table 1). The domain organization of the PKS modules exactly follows the model suggested by the structure of the core macrolactone and the PKS pattern of colinearity. In addition, the acyltransferase (AT) domains 2 and 6 contain the signature motifs associated with the incorporation of malonate.¹⁶ Modules 5 and 11 contain the residues that have been predicted to specify the selection of methoxymalonate,^{17,18} while the remaining extender modules are specific for methylmalonate,¹⁶ again matching the predicted motifs of the bafilomycin structure (see Supporting Information). The AT domain of the loading module was expected to be specific for the incorporation of isobutyrate, suggested by the metabolite structure and confirmed by precursor feeding studies.¹⁹ This domain aligns most closely with the isobutyrate loading domains from the lipomycin^{20, 21} and tautomycin²² pathways. Downstream of the core open reading frames is *bafH*, a PKS type II thioesterase (TEII). Homologues of this enzyme have been found in a number of PKS clusters and are known to possess an editing role during biosynthesis.²³⁻²⁵

The identity of this gene cluster was established by targeted disruption of bafilomycin production using the REDIRECT system.²⁶ When *bafAIII* was replaced in-frame by the apramycin resistance cassette (genotype confirmed by PCR analysis, see Supporting Information), the mutant strain of *S. lohii* failed to produce bafilomycins, as demonstrated by HPLC analysis of culture extracts (Figure 1).

The core macrolactone bafilomycin A₁, presumably formed upon the activity of the TE domain in BafAV, acquires enhanced bioactivity by the addition of a fumarate moiety to the hydroxyl group on C21 to form bafilomycin C₁.² Further potency is provided by a 2-amino-3-hydroxy-cyclopent-2-enone (C₅N) ring attached to the pendant fumaryl group via an amide bond formed with the distal carboxylate resulting in the conversion to bafilomycin B₁ (Scheme 1).

Notably, this C₅N moiety, present in a variety of secondary metabolites,²⁷ often serves as a hydrogen bond donor/accepter pharmacophore. Its biosynthesis has long been proposed to be derived from 5-aminolevulinic acid (5-ALA) via an unusual cyclization process. This putative transformation was recently confirmed in the ECO-02301 pathway (Scheme 2).²⁸ First, the pyridoxal 5-phosphate (PLP) dependent 5-ALA synthase (ALAS) Orf34 catalyzes the condensation of succinyl-CoA and glycine to yield 5-ALA, which is subsequently activated by the acyl-CoA ligase (ACL) Orf35 to form 5-ALA-CoA. Next, Orf34 acts again to cyclize 5-ALA-CoA to afford C₅N, avoiding the non-enzymatic cyclization to the 6-membered ring 2, 5-piperidinedione. Finally, the ATP-dependent amide synthetase (AMS) Orf33 is responsible for linking C₅N to the carboxyl group. Interestingly, this enzyme trio is

similarly encoded by a set of three genes located at the 5' end of the bafilomycin gene cluster, wherein BafX, BafY, and BafZ are 62/72%, 40/55%, 68/80% identical/similar to Orf35, Orf33, and Orf34, respectively, at the protein level. This strongly suggests that the conversion from bafilomycin C₁ to B₁ is likely mediated sequentially by BafZ → BafX → BafZ → BafY (Scheme 2).

To provide evidence for this proposed tailoring pathway, the activity of BafZ was characterized *in vitro*. In addition, this confirmed the proposed functionality of BafZ and validated biochemically the identity of the bafilomycin cluster. It is also expected that BafZ might be useful in industrial fermentation to overproduce 5-ALA, which has been widely used as a photodynamic medicine to treat various cancers, a selective biodegradable herbicide or insecticide, and a precursor for the production of heme-containing enzymes, porphyrin, and vitamin B₁₂.^{29, 30} According to the similarity between BafZ and Orf34, the BafZ catalyzed condensation should be classified as the C₄ pathway (the Shemin pathway) for 5-ALA biosynthesis, which does not exist in *Escherichia coli* (the normally selected industrial strain).³⁰ Thus, the introduction of the *bafZ* gene into *E. coli* would likely supplement its native C₅ pathway of 5-ALA, thereby enhancing the overall production of 5-ALA. The similar strategy using the ALAS gene *hemA* from *Rhodobacter sphaeroides* has already achieved significant improvement of 5-ALA productivity.^{30, 31}

To characterise the *in vitro* activity of BafZ, its encoding gene *bafZ* was PCR amplified from *S. lohii* and cloned into the expression vector *pACYCDuet-1*. The protein was overexpressed in *E. coli* BL21 (DE3) and purified using Ni-NTA column chromatography (see Supporting Information). Upon incubation of purified BafZ (35 μM) with substrates glycine (5 mM) and succinyl-CoA (1 mM) at 28 °C for 2 h, significantly formation of a new compound with retention time and molecular weight (*obs.* 132.0658; *calc.* 132.0655) matching an authentic standard of 5-ALA was detected during HR-LCMS analysis (Figure 2).

In sequencing >15kb upstream and downstream of the defined *baf* cluster, no additional ORFs were identified to encode an enzyme catalyzing attachment of the fumarate moiety. Clustering of all genes involved in the biosynthesis of a given product is the paradigm for *Streptomyces* secondary metabolite production. However, separation of the 2-amino-3-hydroxy-cyclopent-2-enone production genes from those involved in the production of the core polysaccharide in the moenomycin pathway illustrates one of several exceptions to this rule.³² Therefore, this could be the case with the bafilomycin cluster in *S. lohii*. Alternatively, it is possible that the amide synthetase BafY also catalyzes the ester bond formation between fumarate and bafilomycin A₁, which is supported by the recent report that a non-ribosomal peptide synthetase condensation enzyme SgcC5 is capable of catalyzing both amide and ester bond formation.³³ To test these hypotheses, additional work will be necessary, and is currently ongoing in our laboratories.

The incorporation of glycerol-derived precursors has been inferred from feeding studies conducted more than twenty years ago.³⁴ Recently, a number of biosynthetic gene clusters utilizing the glycerol-derived methoxymalonate extender molecule have been sequenced. Associated with these clusters is an operon responsible for the biosynthesis of this uncommon extender unit.^{17, 35, 36} Downstream of *bafAI-A V* are *bafB-F*, five open reading frames predicted to encode enzymes responsible for methoxymalonate biosynthesis (Table 1). BafC is a discrete acyl carrier protein that is loaded with glycerate, presumably oxidized from glycerol during primary metabolism by the enzyme BafE. The subsequent steps in the generation of methoxymalonate are each proposed to be catalyzed as a BafC bound intermediate. First, BafB is proposed to oxidize the 3-hydroxy group into an aldehyde, which is subsequently converted to a carboxylic acid. This conversion is catalyzed by BafD,

an acyl-ACP dehydrogenase.³⁷ Finally, the α -hydroxyl group is methylated by BafF, a putative *O*-methyltransferase. While following a genetic organization conserved within several pathways, each of these genes is most closely related to their discontinuously distributed counterparts found in the concanamycin biosynthetic gene cluster from *Streptomyces neyagawaensis* ATCC 27449.¹⁷

Based on the results of feeding studies, Schumann *et al.* have proposed that methoxymalonate is selected by specific AT domains directly from the dedicated ACP (BafC).¹⁹ Specific protein interaction was inferred due to low incorporation of an *N*-acetylcysteamine thioester (SNAC) of methoxymalonate. However, it is possible that methoxymalonate is transferred to coenzyme A, which then acts as the substrate for the AT. This scenario is more consistent with the classical mechanisms of PKS systems. The enzyme encoded by ORF3 could possibly fulfill this function. It is predicted to be a CoA ligase and shares high homology with an enzyme encoded as part of the concanamycin biosynthetic gene cluster.¹⁷

Downstream of the biosynthesis ORFs are a number of genes encoding putative regulatory elements. BafG is a homologue of AfsR, which has been shown to be a pleiotropic transcriptional activator involved in the regulation of secondary metabolite biosynthesis in the model organism *Streptomyces coelicolor*.³⁸ Homologues of this protein are associated with a number of secondary metabolite biosynthesis pathways within the *Streptomyces* genus including the closely related concanamycin pathway.^{17, 39} Orf1 encodes a member of the LuxR family of transcriptional repressors. Repression is relieved by the binding of a quorum-sensing molecule, most often a homoserine lactone.⁴⁰ LuxR family proteins are associated with a number of secondary metabolite biosynthesis clusters, including the concanamycin system.¹⁷

In summary, sequencing and analysis of the bafilomycin biosynthetic genes represents a novel 16-membered plecomacrolide pathway identified after the elaiophylin gene cluster.⁴¹ Recently, the discovery of more bafilomycin derivatives⁴²⁻⁴⁴ from diverse *Streptomyces* strains and additional putative bafilomycin biosynthetic genes⁴⁵ suggests broad distribution of this biosynthetic system. Thus, the current work provides an important reference for identification and analysis of analogous gene clusters, and crucial information for the future engineered biosynthesis of bafilomycin analogues. Moreover, the *in vitro* characterization of BafZ expands the pool of 5-ALA synthases, which might benefit industrial production of 5-ALA.

Experimental Section

Genomic DNA was isolated from *S. lohii* using standard *Streptomyces* protocols.⁴⁶ A genomic library was constructed in *E. coli* Epi300 using a Fosmid (Epicentre) vector. Initial candidates were selected from a library of 1152 clones by probing with a radiolabelled ketosynthase gene fragment. This probe was a heterologous gene fragment generated by PCR using degenerate KS targeting primers 4UU and 5LL (see Supporting Information) and *S. lohii* gDNA as the template.

Sequence was assembled using Seqman software (DNASTar). Translation analysis was carried out using the MacVector open reading frame analysis function. Functional assignment was accomplished by BLAST analysis¹⁵ against amino acid sequences contained within the NCBI and EMBL databases. Polyketide synthase domain organization was facilitated by the PKS/NRPS database maintained by the Indian National Institute of Immunology.⁴⁷

Gene disruption in *S. lohii* was performed using a modified version of the REDIRECT system.²⁶ A suicide knockout vector was created using fosmid 8D3 from the cluster sequencing effort by replacing the gene of interest with a selectable disruption cassette. This fosmid was transferred by interspecies conjugation from *E. coli* ET12567 into *Streptomyces*. Genetic confirmation of the disruption was accomplished by PCR amplification using genomic DNA as template.

Metabolite profiling of the genetically confirmed knock out strains and one wild-type strain was accomplished by extracting 12 ml cultures grown for 72 hours. The cultures were centrifuged to remove cells and the supernatant was extracted twice with an equal volume of dichloromethane. The organic extracts from each culture were pooled and dried in vacuo, and redissolved in DMSO. Extracts were analyzed by LC-MS on a C-18 column eluted with a gradient of acetonitrile in water with 0.1% formic acid. The mass and retention times were validated by comparison to an authentic standard purchased from Sigma Aldrich.

The *bafZ* gene was amplified from gDNA of *S. lohii* using the primers as follow: forward primer, *cgcggatccgatgaaccattacctggagctcttc tca* (the italic letters indicate the *Bam*HI site); reverse primer, *caatgaagctttcagccgtccgttcggcgaccctgtg* (the underlined letters indicate the *Hind*III site), and inserted into *pACYCDuet-1* (Novagen) between the restriction sites of *Bam*HI and *Hind*III to afford the expression vector *pACYCDuet-1-bafZ*. To prepare BafZ protein, *E.coli* BL21 (DE3) cells carrying *pACYCDuet-1-bafZ* were grown at 37 °C in LB medium containing 25 µg/mL of chloramphenicol until OD₆₀₀ reached ~0.4. Then, isopropyl- β -thiogalactopyranoside (IPTG) was added to the final concentration of 0.1 mM to initiate the protein overexpression. The cells were cultured at 16 °C for another 16 h, collected by centrifugation (5000 g, 10 min, 4 °C), resuspended in 30 mL of lysis buffer (20 mM HEPES, pH 8.0, 0.5 M NaCl, and 5 mM imidazole), and lysed by sonication on ice. After a high-speed centrifugation (40,000 g, 30 min, 4 °C) to remove the cell debris, 1 mL Ni-NTA agarose resin (Qiagen) was added to the supernatant, and mixed at 4 °C for 1 h. Next, the resin with bound proteins was loaded onto a gravity flow column, washed with 50 mL wash buffer (20 mM HEPES, pH 8.0, 0.5 M NaCl, and 20 mM imidazole), and eluted by elution buffer (20 mM HEPES, pH 8.0, 0.5 M NaCl, and 250 mM imidazole). The purified BafZ was buffer exchanged into desalting buffer (50 mM NaH₂PO₄, 10% glycerol) using the PD-10 column (GE Healthcare). The final proteins were flash frozen in liquid nitrogen and stored at -80 °C for the following use.

The *in vitro* BafZ assay was performed by following Zhang *et al.*²⁸ Briefly, 35 µM of purified BafZ was incubated with 5 mM glycine and 1 mM succinyl-CoA in a 100 µl reaction at 28 °C for 2 h; 100 µl of methanol was added to stop the reaction. After removal of the precipitated proteins by centrifugation, the supernatant was subjected to HR-LCMS analysis (Dionex Ultimate 3000 with Bruker maxis Q-TOF) using a BEH Amide column (2.1 mm*100 mm, 1.7 µm, Waters). Acetonitrile (solvent B) and water/5% acetonitrile (solvent A) were used as the mobile phases with a flow rate of 0.20 ml/min. The program was as follow: 90-70% B in A over 10 min, 70% B for 5 min, 70-90% B over 1 min, 90% B for 4 min.

Additional experimental details are available in the Supporting Information. The nucleotide sequence reported within have been deposited in GenBank under the accession number GU390405.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HPLC chromatograms of extracts of wild-type *S. lohii* compared to three separate *bafAIII* gene-disruption mutants.

Figure 2. Extracted ion chromatograms ($m/z=132.06$). A) the negative control with boiled BafZ added to the reaction; B) the BafZ-catalyzed synthesis of 5-ALA from succinyl-CoA and glycine; C) 5-ALA authentic standard. The high resolution mass spectrum of 5-ALA is shown in Supporting Information.

Scheme 1

A) The organization of open reading frames in the bafilomycin biosynthetic gene cluster. B) The domain organization of the type I polyketide synthases encoded by ORFs BafAI through BafAV.

Scheme 2

Putative pathway for 5-ALA biosynthesis and attachment.

Table 1

Predicted Functions of the Open Reading Frames Show in Scheme 1.

Protein	Amino Acids	Putative Function	Nearest Homolog (Enzyme, Origin)	Identity/Similarity	Accession Number
BafX	528	acyl CoA ligase	MoeA4 [<i>Streptomyces ghanaensis</i>]	72%/83%	ZP_06574361.1
BafY	514	amide synthetase	Amide synthetase[<i>Streptomyces tsukubaensis</i>]	78%/85%	ZP_10067139.1
BafZ	414	5-aminolevulinic acid synthase	aminolevulinic synthase [<i>Streptomyces nodosus</i> subsp. <i>asukaensis</i>]	73%/83%	AAO62615.1
BafAI	4884	PKS loading module + modules 1-3	polyketide synthase [<i>Streptomyces neyagawaensis</i>]	62%/70%	AAZ94387.1
BafAII	5145	PKS modules 4-6	BFAS2 [<i>Streptomyces tsukubaensis</i>]	78%/83%	ZP_10067132.1
BafAIII	3968	PKS modules 7,8	BFAS2 [<i>Streptomyces tsukubaensis</i>]	79%/85%	ZP_10067131.1
BafAIV	3511	PKS modules 9,10	polyketide synthase [<i>Streptomyces scabiei</i>]	62%/73%	YP_003493865.1
BafAV	2103	PKS module 11 + Thioesterase	polyketide synthase [<i>Streptomyces clavuligerus</i>]	61%/69%	ZP_06769488.1
BafB	296	glyceryl-ACP oxidase	methoxymalonate biosynthesis protein [<i>Streptomyces tsukubaensis</i>]	72%/81%	ZP_10067126.1
BafC	93	acyl carrier protein (ACP)	GalJ [<i>Streptomyces galbus</i>]	69%/80%	ADE22334.1
BafD	363	acyl-ACP dehydrogenase	methoxymalonate biosynthesis protein [<i>Streptomyces tsukubaensis</i>]	85%/93%	ZP_10067124.1
BafE	365	glycerate ACP ligase	methoxymalonate biosynthesis protein [<i>Streptomyces tsukubaensis</i>]	83%/90%	ZP_10067123.1
BafF	220	O-methyl transferase	O-methyltransferase mdmC [<i>Streptomyces clavuligerus</i>]	73%/81%	ZP_05008783.1
BafG	609	AfsR homolog	putative regulator [<i>Streptomyces neyagawaensis</i>]	54%/64%	AAZ94408.1
BafH	253	TEII	thioesterase [<i>Streptomyces auratus</i>]	65%/76%	ZP_10551360.1
ORF1	117	LuxR homolog	LuxR family transcriptional regulator [<i>Streptomyces tsukubaensis</i>]	65%/73%	ZP_10067119.1
ORF2	320	Putative Malonyl transferase	acyl-carrier-protein S-malonyltransferase [<i>Streptomyces tsukubaensis</i>]	84%/90%	ZP_10067118.1
ORF3	332	Putative CoA ligase	hypothetical protein [<i>Streptomyces neyagawaensis</i>]	74%/83%	AAZ94384.1