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Structure and Biosynthesis of Heat-Stable Antifungal Factor (HSAF), a Broad-Spectrum Antimycotic with a Novel Mode of Action

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A screen for antifungal compounds from Lysobacter enzymogenes strain C3, a bacterial biological control agent of fungal diseases, has previously led to the isolation of heat-stable antifungal factor (HSAF). HSAF exhibits inhibitory activities against a wide range of fungal species and shows a novel mode of antifungal action by disrupting the biosynthesis of a distinct group of sphingolipids. We have now determined the chemical structure of HSAF, which is identical to that of dihydroxymaltolphilin, an antifungal metabolite with a unique macroyclic lactam system containing a tetramic acid moiety and a 5,5,6-tricyclic skeleton. We have also identified the genetic locus responsible for the biosynthesis of HSAF in strain C3. DNA sequencing of this locus revealed genes for a hybrid polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS), a sterol desaturase, a ferredoxin reductase, and an arginase. The disruption of the PKS-NRPS gene generated C3 mutants that lost the ability to produce HSAF and to inhibit fungal growth, demonstrating a hybrid PKS-NRPS that catalyzed the biosynthesis of the unique macrolactam system that is found in many biologically active natural products isolated from marine organisms. In addition, we have generated mutants with disrupted sterol desaturase, ferredoxin reductase, and arginase and examined the metabolites produced in these mutants. The work represents the first study of the genetic basis for the biosynthesis of the tetramic acid-containing macrolactams. The elucidation of the chemical structure of HSAF and the identification of the genetic locus for its biosynthesis establish the foundation for future exploitation of this group of compounds as new fungicides or antifungal drugs.

Heat-stable antifungal factor (HSAF) is a secondary metabolite produced by the bacterium Lysobacter enzymogenes strain C3 (originally called Stenotrophomonas maltophilia strain C3 [24]), a biological control agent originally isolated from grass foliage (7). Strain C3 was demonstrated in the field to reduce the foliage (24), a biological control agent originally isolated from grass foliage (7). Strain C3 was demonstrated in the field to reduce diseases caused by multiple fungal pathogens, including Rhizoctonia solani (27). It was also found to be effective in inhibiting the soilborne pathogens Pythium ultimum (12) and Magnaporthe poae (13) in greenhouse experiments. A search for antifungal factors in strain C3 led to the isolation of HSAF, which exhibits strong activity against a wide range of fungi (15).

The mechanism by which HSAF inhibits the growth of Aspergillus nidulans has been studied (16). HSAF disrupts the polarized growth of the fungus. Genetic analysis of A. nidulans mutants suggests that HSAF targets the biosynthesis of sphingolipids (16), which are ubiquitous components of eukaryotic cell membranes and signaling molecules involved in numerous cellular processes. Interestingly, HSAF appears to target a distinct group of sphingolipids that are required for polarized growth of filamentous fungi and appears to be absent from mammals and plants. Therefore, the antifungal mechanism of HSAF represents an unprecedented mode of action for an antifungal metabolite, and thus, the study of HSAF could lead to the development of novel fungicides or antifungal drugs.

The objectives of this study were to determine the chemical structure of HSAF and to identify the genetic locus in strain C3 that is required for the production of HSAF. The outcomes of the study will provide important knowledge for future application of HSAF and possible genetic engineering of the biosynthetic pathway to improve the production of HSAF or to produce new analogs.

MATERIALS AND METHODS

Bacterial and fungal strains, plasmids, and general DNA manipulations. L. enzymogenes strain C3 and other bacterial strains were grown in Luria-Bertani (LB) broth medium or 1/10-strength tryptic soy broth (1/10 TSB; Sigma) at 28°C with shaking. Escherichia coli strain DH5α was used as the host for general DNA propagations. E. coli S17-1 was obtained from D. Y. Kobayashi (Rutgers University, New Brunswick, NJ). The plasmids used for cloning and sequencing included the pGEM-z series from Promega (Madison, WI) and pANT841 (21). Plasmid preparation and DNA gel extraction were carried out with commercial kits (Qiagen, Valencia, CA), and all other manipulations were performed by standard methods (22). The genomic DNA of L. enzymogenes strain C3 was prepared as described previously (12).

Cloning of genes for HSAF biosynthesis. Genomic DNA was prepared from a polyketide synthase (PKS)-disrupted C3 mutant (see below). Approximately 10 μg of the DNA was digested with 50 units of PstI in a total volume of 50 μl. The PstI-digested DNA was precipitated by ethanol and resuspended in 20 μl H2O. The ligation reaction was performed by mixing 8 μl of the digested DNA with 1 μl of ligation buffer and 1 μl of T4 DNA ligase (5 units; Promega), and the mixture was incubated at 15°C for 24 h. An aliquot of 5 μl of the ligation mixture was used to transform E. coli DH5α, and the cells were spread onto LB plates containing 20 μg/ml gentamicin. Plasmid DNA was prepared from the developed colonies and screened by PCR assays with primers P1 and P2 (see below and Fig. 2). One of the rescued plasmids, pJQ208SK-5.3KB, which harbors a 5.3-kb DNA fragment, was
screened. Similarly, the genomic DNA was digested with BamHI, SaI, or SacI, and individual plasmids were rescued upon self-ligation. The sequencing of these plasmids revealed an approximately 13-kb DNA region.

**Construction of gene disruption vectors.** To construct the PKS disruption vector, two PCR primers, primers P1 (CCGCGGAAGTCGAAGCAGTA) and P2 (AAGGACTGCTCCTACCGAC), were designed on the basis of the preliminary data reported in Folman’s Ph.D. dissertation (6). Folman obtained an 818-bp DNA fragment from a minitransposon (mini-Tn5) mutant of *L. enzymogenes* strain C3. The transposon insertion led to the loss of antifungal enzymogenes CC). This fragment was cloned into the pGEM-T vector. The PCR product was sequenced. Similarly, the genomic DNA was digested with BamHI, SaI, or SacI, and individual plasmids were rescued upon self-ligation. The sequencing of these plasmids revealed an approximately 13-kb DNA region.

**Figure 1.** Structures of HSAF and several tetramic acid-containing macrolactams.

**Figure 2.** Scheme for PKS gene disruption in *L. enzymogenes* strain C3. A 407-bp fragment of the gene (shaded bar) was amplified from the genomic DNA and cloned into a gene disruption vector to produce pJQ200SK-PKS. 

**Conjugational transfer of pJQ200SK constructs from E. coli S17-1 to *L. enzymogenes* strain C3.** pJQ200SK constructs were first introduced into *E. coli* S17-1 and then conjugally transferred from strain S17-1 to strain C3. To conjugally transfer the PKS-disrupted mutants, genomic DNA was prepared from LB cultures of the individual candidates. For the PKS-disrupted mutants, primer P1 (GGTGTCGATCGACAGTCC) and primer P2 (AAGGACTGCTCCTACCGAC) were used for PCR. Primers P3 and P4 anneal to the flanking regions of the 407-bp fragment used in the homologous recombination (see Fig. 2). Thus, only the mutants that result from a homologous recombination produce a 453-bp PCR fragment when primers P3 and T3 are used and a 522-bp fragment when primers T7 and T4 are used. The

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**Summary:**

- Construct PKS disruption vector using PCR with primers P1 and P2.
- Use SaI or SacI digests to clone the fragment into T vector for confirmation.
- Use SaI digestion from a subclone to release the gene for cloning.
- Screen for gene disruption mutants using PCR to amplify fragment and screening.
- Conjugate constructs from E. coli S17-1 to *L. enzymogenes* strain C3.
- Use primers P3 and P4 for homologous recombination to identify disrupted genes.

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**Notes:**

- Figure 1 includes structures of HSAF and several tetramic acid-containing macrolactams.
- Figure 2 shows a scheme for PKS gene disruption in *L. enzymogenes* strain C3, with a 407-bp fragment of the gene cloned into a vector and used for conjugation.
- The text describes the experimental methods for gene disruption, including PCR amplification, cloning, and screening.

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**Additional Information:**

- The text provides a detailed description of the experimental procedures and results, including DNA digestion, cloning, PCR, and screening.
- It is important to note that the text is a continuous narrative, and each section builds upon the previous one, providing a comprehensive understanding of the experimental methods and results.

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**References:**

- Folman’s Ph.D. dissertation (6)
- Preliminary data reported in Folman’s Ph.D. dissertation (6)

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**Acknowledgments:**

The authors acknowledge the contribution of Folman’s Ph.D. dissertation (6) and preliminary data reported therein.

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**Conclusion:**

The study described in the text provides a detailed and comprehensive approach to constructing gene disruption vectors for *L. enzymogenes* strain C3, using PCR and homologous recombination. The results are significant for understanding the biosynthesis of HSAF and its tetramic acid-containing macrolactams.
wild type or mutants that resulted from random insertion did not produce any of the 
PCR fragments. To identify the NPRS-disrupted mutants, PCR was carried 
out with primer N1 (ACGCCAGGCTCGAAAATACAC) and primer N2 (ATC 
GAGACTTCCGCAACAG). The expected size of the PCR product is 695 bp, 
and this product produces two fragments of 225 bp and 470 bp upon SphI 
digestion. To identify the ferredoxin reductase gene-disrupted mutants, PCR 
was carried out with primer Ferr-1 (ACGACAGCCTTTTCAAGCTTC) and primer 
T7. The expected size of the PCR product is 661 bp, and this product produces two fragments of 209 bp and 452 bp upon SacI digestion and two fragments of 
272 bp and 389 bp upon Smal digestion. To identify the sterol desaturase 
gene-disrupted mutants, PCR was performed with primer T7 and primer S5 
(TGAAACCGATTATCAGAGCAC). The expected product is a 957-bp fragment 
that produces three fragments of 670 bp, 171 bp, and 116 bp upon SphI digestion. 
To identify the arginase gene-disrupted mutants, PCR was conducted with 
primer T7 and primer Arg1 (GTTCGCATCCACCTCTTTTTC). The expected 
size is 606 bp, and this product produces two fragments of 294 bp and 312 bp 
upon Sall digestion.

**Isolation and HPLC analysis of metabolites from strain C3 and mutants.**

Strain C3 and individual mutant strains were cultured in 1/10 TSB (supple-
mented with 20 μg/ml gentamicin for mutant strains) at 28°C in a rotary shaker 
at 180 rpm for 4 days. The supernatant was collected from each culture by 
centrifugation at 10,000 × g at 4°C for 30 min. To analyze the metabolites 
excreted into the culture, the supernatant (10 to 20 μl) was directly injected into 
a high-pressure liquid chromatograph. A high-pressure liquid chromatography 
(HPLC) system (ProStar, model 210; Varian, Walnut Creek, CA) with an 
Alltima C18LL column (250 mm by 4.6 mm [inner diameter]; 5 μm; Alltech, 
Deerfield, IL). The mobile phases were water-trifluoroacetic acid (TFA; 100: 
0.025; vol/vol) (mobile phase A) and acetonitrile-TFA (100:0.025; vol/vol) 
(mobile phase B) with a gradient of 0 to 40% mobile phase B in mobile phase 
A in the first 10 min, 40 to 80% mobile phase B in mobile phase A from 10 to 15 min, 
80% mobile phase B in mobile phase A from 15 to 20 min, 80 to 100% mobile 
phase B in mobile phase A from 20 to 21 min, 100% mobile phase B in mobile 
phase A from 21 to 23 min, and 100 to 0% mobile phase B in mobile phase A 
from 23 to 25 min. The flow rate was 1.0 ml/min. The peaks were detected at 220 
nm on a UV-visible detector (ProStar, model 310; Varian). To prepare HSAF 
and its analogs for structural analysis, ammonium sulfate was added to the 
supernatant of the culture, with constant stirring until the final concentration 
reached 0.5 g/ml. The solution was incubated at 4°C for 12 h. The precipitate 
was collected by centrifugation at 10,000 × g at 4°C for 25 min and was dissolved 
in 100% methanol. The methanol fraction containing HSAF or its analogs was 
centrifuged at 10,000 × g at 25°C for 10 min, and the resultant supernatant 
was collected and dried with a Rotavapor. The residues were redissolved in 10% 
acetonitrile, and the solution was loaded on a Sep-Pak Vac 35-ml (10-g) C18 
cartridge (Waters, Milford, MA) previously conditioned with 200 ml of 100% 
acetonitrile, followed by 200 ml of 10% acetonitrile. The cartridge was sequen-
tially washed with 100 ml of 10% acetonitrile and 100 ml of 20% acetonitrile, and 
HSAF was eluted with 100 ml of 30% acetonitrile. HSAF was further purified by 
preparative HPLC (250 mm by 10 mm [inner diameter]: 5-μm) column with the 
same system described above. The fractions containing HSAF were combined, 
and the final pure HSAF was obtained by crystallization in methanol.

**HR-MS and NMR analysis of the metabolites.**

The melting point was measured with a Thomas-Hoover capillary melting point apparatus and was uncor-
corrected. High-resolution electron-spray ionization mass spectrometry (MS) of the 
purified HSAF was performed on an API Qtrap 4000 instrument in CD3OD and were calibrated by using the solvent peak (m/z 3.31) as 
an internal reference.

**Biological assays for antifungal activity.** One milliliter of the culture broth of 
the mutants or the wild type was collected and centrifuged on a bench-top 
centrifuge at 16,000 × g for 10 min. The supernatant was transferred to a new 
tube and was boiled for 10 min in a water bath. The sample was centrifuged at 

![FIG. 4. HPLC analysis and antifungal activity assay for the PKS-disrupted mutants. (A and B) HPLC traces of culture broths from mutant 
strain K19 (A) and wild type (B), with the peak of HSAF indicated; (C and D) activity assay for the culture broth from mutant strain K19 (C) 
and the wild type (D) by using Bipolaris sorokiniana as the test organism.](image-url)
The methanol solution was first applied onto a slide glass and air-dried. One of the preparations was dried in a Speed-Vac and redissolved in 100% methanol. For the bioassays of purified HSAF and its analogs, one- and two-dimensional NMR. High-resolution electron-spray ionization mass spectrometry gave an m/z 513.2954 for [M(C_{29}H_{40}O_{6}N_{2}) + H]^+ (calculated, 513.2959), which is 2 mass units heavier than the mass of the reference compound, maltophilin (purchased from EMC Microcollections GmbH, Tubingen, Germany) (Fig. 1). HSAF appeared as a colorless powder with a melting point of greater than 300°C when it was crystallized from methanol. 1H-NMR (in CD_{3}OD) gave the following data: δ 8.02 (1H, br s, OH or NH, disappeared immediately after the addition of D_{2}O or after the sample was kept standing for 2 days), 7.13 (1H, d, J = 13.2 Hz, H-10), 6.65 (1H, br dd, H-11), 6.05 (1H, br t, J = 10.0 Hz, H-25), 5.80 (1H, br d, J = 12.0 Hz, H-26), 3.97 (1H, br d, J = 6.0 Hz, H-4), 3.92 (1H, br s, H-5), 3.48 (2H, m, H-2a,24a), 3.36 (1H, m, H-14), 2.79 (1H, br t, J = 12.4 Hz, H-2b), 2.45 (1H, m, H-20), 2.08 to 2.19 (4H, m, H-12, 19a, 21a, 24b), 1.86 to 1.92 (2H, m, H-13a, 16), 1.72 (1H, m, H-22), 1.63 (2H, m, H-3a, 30a), 1.29 to 1.36 (5H, m, H-3b, 13b, 17, 18, 23), 1.23 (1H, q, J = 10.0 Hz H-15), 1.11 (3H, d, J = 6.4 Hz, H-29), 1.10 (1H, overlapping, H-30b), 0.96 (1H, m, H-21b), 0.90 (3H, t, J = 7.4 Hz, H-31), 0.86 (1H, overlapping, H-19b). These assignments, which were based on the results of the 1H-1H correlation spectroscopy experiment, are consistent with the reported values for dihydromaltophilin recorded in CD_{3}OD-CDCl_{3} (3:1) (8). The coupling constants, which were well resolved for individual protons, are presented here, since the previous paper did not report these values. These data show that HSAF isolated from \textit{L. enzymogenes} strain C3 has a chemical structure identical to that of dihydromaltophilin (Fig. 1), which was isolated from a \textit{Streptomyces} species (8).

PKS-disrupted mutants lost the ability to produce HSAF and to inhibit fungal growth. In order to identify the genetic locus required for the biosynthesis of HSAF in strain C3, we
generated mutants through target-specific gene disruption by taking advantage of the partial sequence for a putative PKS gene isolated from L. enzymogenes strain 3.1T8 (6). Using two primers based on a region in the reported sequence, we amplified a 407-bp PCR product from the genomic DNA of strain C3. The DNA sequence of the fragment from strain C3 was 96.6% identical to that from strain 3.1T8. It also suggested that the 818-bp sequence reported in the earlier study likely contained sequence errors that resulted in frame shifts.

To determine if the putative PKS gene is involved in HSAF biosynthesis, we constructed a gene disruption vector, pJQ200SK-PKS, using the fragment amplified by PCR. Homologous recombination of the fragment with the corresponding region in the chromosome of C3 should generate PKS-disrupted mutants (Fig. 2). After mating of E. coli S17-1 carrying pJQ200SK-PKS with strain C3, 22 colonies developed on selection medium containing gentamicin and kanamycin. Among the colonies, 12 resulted from homologous recombination, as confirmed by PCR analysis. A 522-bp fragment was amplified from the genomic DNA prepared from the individual colonies by using primer T7 and primer P4 (Fig. 3), whereas a 453-bp fragment was obtained when primers P3 and T3 were used (data not shown). No PCR product was obtained from the wild type or the remaining 10 colonies, which presumably resulted from random insertion of the vector. The PCR products were sequenced, and the result confirmed that the colonies were PKS-disrupted mutants (Fig. 2).

The PKS-disrupted mutants were examined for their antifungal activities and HSAF production. While the boiled culture broth of wild-type strain C3 completely inhibited spore germination in B. sorokiniana, the boiled culture broth of the PKS-disrupted mutants did not inhibit spore germination (Fig. 4). The supernatants from the mutant and the wild-type cultures were further analyzed by HPLC. The supernatant from the wild type exhibited a peak at 19.2 min that corresponded to HSAF, whereas the mutants did not produce HSAF (Fig. 4). The results demonstrate that the putative PKS gene is required for the biosynthesis of HSAF.

**HSAF biosynthetic genes.** Following digestion of genomic DNA from the PKS-disrupted mutants with several restriction enzymes and rescue of the vector pJQ200SK by self-ligation, we sequenced the regions flanking the inserted vector. Sequences totaling about 13.5 kb were determined, and the analysis of the sequences indicated that this region contains four open reading frames: those for a hybrid polyketide synthase-nonribosomal peptide synthetase, a sterol desaturase, a ferredoxin reductase, and an arginase (Fig. 5). The hybrid PKS-NRPS contains nine domains: those for ketosynthase, acyltransferase, dehydratase, ketoreductase, acyl carrier protein, condensation, adenylation, peptidyl carrier protein, and thioesterase. All the highly conserved signature motifs within the domains were readily identifiable (Fig. 5). The PKS-NRPS contains 3,124 amino acid residues and is most similar (59% identity and 74% similarity from the start to the end) to the PKS-NRPS sequence from Saccharophagus degradans 2-40 (GenBank accession number YP_529192). The sequence also showed that the partial sequence of the putative PKS gene from strain 3.1T8 reported by Folman (6) is located at the ketosynthase domain of the hybrid PKS-NRPS.

**Function of PKS-NRPS gene in HSAF synthesis.** To further confirm the role of the predicted hybrid PKS-NRPS in HSAF biosynthesis, we generated mutants with a disrupted NRPS module (at the C-domain region). A total of 32 putative NRPS-disrupted mutants were produced, 20 of which were confirmed by PCR assays with primer N1 and primer N2. A 695-bp fragment was amplified from the strains, and the fragment was cleaved by SphI to produce two fragments of 225 bp and 470 bp, as expected, from the mutants (data not shown). The PCR product was sequenced to further confirm the identities of the mutants. HPLC analysis of the culture broth of four NRPS-disrupted mutants showed that none of the mutants produced HSAF (Fig. 6B). Furthermore, none of the cultures had activity against B. sorokiniana in the spore germination assay (data not shown).

**Functions of ferredoxin reductase, arginase, and sterol desaturase genes.** Three, 12, and 7 mutant strains with disruptions in the ferredoxin reductase, arginase, and sterol desaturase genes, respectively, were generated by mating of wild-type C3 with E. coli S17-1 carrying the respective pJQ200SK-gene construct. Insertion of the vector via homologous recombination into the target gene was confirmed in these strains by PCR analysis (data not shown). The mutants were evaluated for

![Image](https://example.com/image.jpg)
antifungal activities by the spore germination assay. Boiled culture broths from all of the mutant strains had inhibitory effects on the germination of \textit{B. sorokiniana} spores similar to those of the broth from wild-type C3 strain and purified HSAF (data not shown). HPLC analysis of the broth from cultures offerredoxin reductase- and arginase-disrupted mutants indicated that all of the mutants produced HSAF (retention time, 19.2 min), with the yields being similar to that of the wild type (Fig. 6C and D). This result was confirmed by liquid chromatography-MS, which showed that the compound produced by the mutants had the same molecular weight as HSAF (data not shown). Culture broth from the sterol desaturase-disrupted mutants did not contain HSAF but contained two dramatically increased metabolites (retention times, 19.9 and 20.4 min, respectively) (Fig. 6E). Activity tests showed that both metabolites were active in antifungal assays (data not shown). These two metabolites were also present in the mutants of ferredoxin reductase and arginase, as well as in the wild type, but at a much lower level. Interestingly, none of the PKS-NRPS-disrupted mutants produced these metabolites (Fig. 4B and Fig. 6B), indicating that these are probably precursors or shunt products of HSAF.

**DISCUSSION**

In this study, we identified the primary compound comprising the heat-stable antifungal activity in strain C3 to be dihydromaltophilin, an antibiotic first reported in a \textit{Streptomyces} sp. (8) and an analog of xanthobaccins (17). Dihydromaltophilin belongs to a group of tetramic acid-containing macro lactam natural products, including the marine natural products cylindramide A (11), discodermide (9), and alteramide A (23) (Fig. 1). This group of metabolites has unique structural features and diverse biological activities, including anticancer, antibiotic, antiprotozoal, and antioxidant activities; and there has been extensive research interest in chemical total synthesis to prepare the scarce natural products (2–4, 10, 18). Our study represents the first attempt to investigate the molecular genetic basis for the biosynthesis of this group of natural products.

We were successful in identifying the genetic locus for HSAF biosynthesis in strain C3. The search was facilitated by the report of a putative PKS gene sequence in strain 3.1T8 (6). The high degree of sequence identity (96%) between the corresponding PKS gene regions in strains C3 and 3.1T8 and the fact that disruption of the PKS gene function in both strains resulted in the loss of antifungal activity suggest that strain 3.1T8 could also produce HSAF. Whether or not HSAF production is a common trait among strains of \textit{L. enzymogenes}, however, is unknown. The absence of HSAF production in our PKS-NRPS-disrupted mutants demonstrates that the PKS-NRPS gene is required for the production of HSAF in strain C3. The result also implies
that the biosynthesis of HSAF involves a hybrid polyketide-peptide mechanism.

Since the biosynthetic genes for secondary metabolites in microorganisms are generally found to cluster together at the same locus on a chromosome, we first used the PKS fragment as a probe to screen a genomic library based on the cosmid pLAFR3 (12) in our initial attempt to obtain the complete sequence for the PKS gene, as well as other genes required for HSAF biosynthesis. However, an extensive search of the library did not produce any positive clones, while the control experiments with a known ABC transporter as a probe did successfully identify three positive clones from the same library (data not shown). Subsequently, we constructed a new genomic library based on the cosmid SuperCos 1 (Stratagene). Again, using the PKS fragment as a probe, we isolated four positive clones from the new library, but none of the cosmid clones was stable in the host. The insert DNA in the cosmid vector was found to be either partially lost or rearranged during the regeneration and subcloning process. These results indicate that the HSAF biosynthetic genes might be toxic when they are expressed in the host. Since the direct screening of the genomic libraries did not lead to the identification of the biosynthetic genes, we took an alternative approach. We used the genomic DNA isolated from the gene-disrupted mutants to rescue plasmids that contained the homologous region as well as the flanking regions. The sequencing of the flanking regions eventually led to the identification of a gene cluster encoding a hybrid PKS-NRPS, a sterol desaturase, a ferredoxin reductase, and an arginase.

The chemical structure of HSAF suggests that an ornithine residue is incorporated into the lactam functionalities and that the biosynthesis of HSAF could involve both polyketide and nonribosomal peptide mechanisms, as seen in bleomycins and other natural products (5). This is supported by the identification of a hybrid PKS-NRPS gene. Indeed, a disruption of the NRPS module also led to the elimination of HSAF production in strain C3. This is direct evidence supporting the notion that the unique structure of the tetramic acid-containing macrolactam is derived from hybrid nonribosomal peptide and polyketide biosynthesis. A
careful analysis of the adenylation domain of the NRPS module suggested that this domain is most similar to the ornithine-activating domain of gramicidin S-synthetase 2 from *Brevibacillus brevis* (14). Based on this information, we propose a possible biosynthetic pathway for HSAF (Fig. 7). Two PKS-synthesized hexaketide chains are condensed with the NRPS-activated ornithine via Claisen-type condensations to generate the basic skeleton of this group of natural products. Following a series of aldol condensations, reductions, and oxidations, the 5,5,6-membered tricyclic ring system is formed and fused to the tetramic acid-containing macrolactam ring system (Fig. 7). The different patterns of ring fusion within this group of natural products are intriguing (Fig. 1). For example, HSAF and discodermide have a three-ring (5,5,6-membered) system fused to the macrolactam, while ikarugamycin has a different pattern (5,6,5-membered system [structure not shown]) of the ring system fused to the macrolactam system. On the other hand, cylindramide and alteramidine A have only a two-ring (5,5-membered) system fused to a larger lactam system (Fig. 1). It will be extremely interesting to exploit the properties of the enzymes involved in these post-PKS folding processes. As suggested by this biosynthetic pathway, multiple PKS modules are likely involved in the assembly of the two polyketide chains. The four-domain PKS module of the hybrid PKS-NRPS gene described in this study could catalyze the incorporation of the last two-carbon unit (C<sub>26</sub>-C<sub>27</sub>; Fig. 7) of the first polyketide chain. This is supported by the fact that this module lacks an enoylreductase domain, thus generating a first polyketide chain. This is supported by the fact that this FNR gene in the HSAF gene cluster did not affect HSAF production. However, current data do not exclude the possibility that this FNR is not directly involved in HSAF biosynthesis.

Finally, sterol desaturase catalyzes the dehydrogenation reaction to introduce a carbon-carbon double bond into the sterol ring system, such as sterol C<sub>25</sub>-desaturase for a double bond at C<sub>5</sub>-C<sub>6</sub> (25). Several oxidoreductions are implied in the biosynthesis of HSAF, and the sterol desaturase may play a role in the reactions. The disruption of this gene led to the elimination of HSAF production and a significant increase in the levels of two metabolites (Fig. 6). These two metabolites were also produced by the wild-type strain at a lower level, but their production, along with that of HSAF, was eliminated by the disruption of the PKS-NRPS gene. These results suggest that the metabolites may be precursors of HSAF and that the sterol desaturase is involved in the conversion of the metabolites to HSAF and its analogs.

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