# Microbisporicin gene cluster reveals unusual features of lantibiotic biosynthesis in actinomycetes

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Communicated by Arnold L. Demain, Drew University, Madison, NJ, June 13, 2010 (received for review April 29, 2010)

**Lantibiotics are ribosomally synthesized, posttranslationally modified peptide antibiotics. The biosynthetic gene cluster for microbisporicin, a potent lantibiotic produced by the actinomycete *Microbispora corallina* containing chlorinated tryptophan and dihydroxyproline residues, was identified by genome scanning and isolated from an *M. corallina* cosmid library. Heterologous expression in *Nonomuraea* sp. ATCC 39727 confirmed that all of the genes required for microbisporicin biosynthesis were present in the cluster. Deletion, in *M. corallina*, of the gene (*mibA*) predicted to encode the prepropeptide abolished microbisporicin production. Further deletion analysis revealed insights into the biosynthesis of this unusual and potentially clinically useful lantibiotic, shedding light on mechanisms of regulation and self-resistance. In particular, we report an example of the involvement of a tryptophan halogenase in the modification of a ribosomally synthesized peptide and the pathway-specific regulation of an antibiotic biosynthetic gene cluster by an extracytoplasmic function σ factor–anti-σ factor complex.**

antibiotic | extracytoplasmic function | halogenase | *Microbispora* | peptide | σ factor

Lantibiotics are ribosomally synthesized, posttranslationally modified peptide antibiotics produced by Gram-positive bacteria. They are characterized by lanthionine and methyl lanthionine bridges that give lantibiotics their characteristic conformations and stability (1).

Lantibiotics are encoded by a structural gene (generically named *lanA*) that encodes a prepropeptide with an N-terminal leader peptide followed by the region that will become the mature lantibiotic (the propeptide). The prepropeptide undergoes modification and processing before the mature product is exported from the cell, coincident with, or followed by, leader peptide removal. The N-terminal leader sequence may have roles in directing export, retaining the prelantibiotic in an inactive state until export and recruiting modifying enzymes (1).

Formation of lanthionine bridges occurs via the dehydration of serine and threonine residues, followed by cyclization with cysteine residues. In type AI lantibiotics (e.g., nisin), dehydration is carried out by a LanB enzyme and cyclization by LanC. In type AII (e.g., lacticin 481) and B (e.g., cinnamycin) lantibiotics, a bifunctional LanM carries out both of these reactions. Formation of C-terminal *S*-[(*Z*)-2-aminovinyl]-D-cysteine requires a LanD enzyme. Additional genes, such as those involved in pathway-specific regulation, lantibiotic export, and producer cell immunity, are also found in lantibiotic gene clusters (1).

Strains of the species *Microbispora corallina* (2) produce a group of potent bactericidal lantibiotic variants collectively named microbisporicin (3–5) (Fig. 1*A*). Microbisporicin has one methyllanthionine and three lanthionine bridges and an *S*-[(*Z*)-2-aminovinyl]-D-cysteine at its C terminus. It also contains two unusual lantibiotic modifications: 5-chlorotryptophan and 3, 4-dihydroxyproline (4, 5).

The increasing incidence of antibiotic-resistant bacterial pathogens has resulted in an urgent need for new, clinically useful antibiotics. Microbisporicin is active against a wide range of Gram-positive bacterial pathogens, selectively inhibiting peptidoglycan synthesis in *Staphylococcus aureus* and *Bacillus megaterium*, probably by binding to the immediate precursor for cell wall biosynthesis, lipid II (4, 5). It is active against methicillin-resistant and vancomycin-intermediate resistant strains of S. *aureus* and, unusually for a lantibiotic, also against some Gram-negative species (4, 5). Microbisporicin, under the commercial name NAI-107, is currently in late preclinical-phase trials and has demonstrated superior efficacy in animal models of multidrug resistant infections compared with the drugs of last resort, linezolid and vancomycin.* Interestingly, no microbisporicin-resistant mutants were observed during these studies.† Understanding how microbisporicin is made could enable the development of variants with improved clinical activity.

Few examples of actinomycete lantibiotic gene clusters exist. Those that do (cinnamycin and actagardine) belong to type B lantibiotics (6, 7). Our aim was to clone and characterize the type AI microbisporicin biosynthetic gene cluster from *M. corallina* NRRL 30420 and to develop *M. corallina* as a genetic system in which targeted mutagenesis could be performed to elucidate the functions of individual genes. There were no reported methods by which *M. corallina*, a so-called rare actinomycete, could be transformed with DNA, and only a few reports of transformation in the *Streptosporangiaceae*, the family to which *M. corallina* belongs. To complement genetic analysis in *M. corallina*, we also set out to express the microbisporicin gene cluster in a heterologous host and to define the minimal gene set required for microbisporicin production.

## Results

**Identification of the Microbisporicin Gene Cluster by Genome Scanning.** Genome scanning was used to identify genes from the microbisporicin gene cluster. *M. corallina* genomic DNA (gDNA) was subjected to 454 sequencing (Cogenics), yielding 4.6 Mb of sequence on 7,580 assembled contigs. Sequence analysis identified a contig encoding homologs of LanC and LanD proteins from other lantibiotic gene clusters. Other contigs encoded proteins homologous to the prepropeptide (LanA) of microbisporicin [deduced from the NMR structure of microbisporcin (4, 5)], a putative lantibiotic dehydratase (LanB), and a tryptophan halogenase [with homology to PyrH from *Streptomyces rugosporus* (8)]. Many of these fragments were bridged by PCR amplification

Author contributions: L.C.F. and M.J.B. designed research; L.C.F. performed research; L.C.F. and M.J.B. analyzed data; and L.C.F. and M.J.B. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. HM536998).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008285107/-/DCSupplemental.

*Jabes D, Candiani G, Romano G, Brunati C, Riva S (2009) NAI-107: Superior efficacy in animal models of MDR Gram-positive infections. 49th Annual Meeting of Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12–15, 2009, San Francisco, CA, F1-1503 (abstr).

†Jabes D, Brunati C, Guglierame S, Donadio S (2009) *In vitro* antibacterial profile of the new lantibiotic NAI-107. 49th Annual Meeting of Interscience Conference on Antimicrobial Agents and Chemotherapy, September 12–15, 2009, San Francisco, CA, F1-1502 (abstr).





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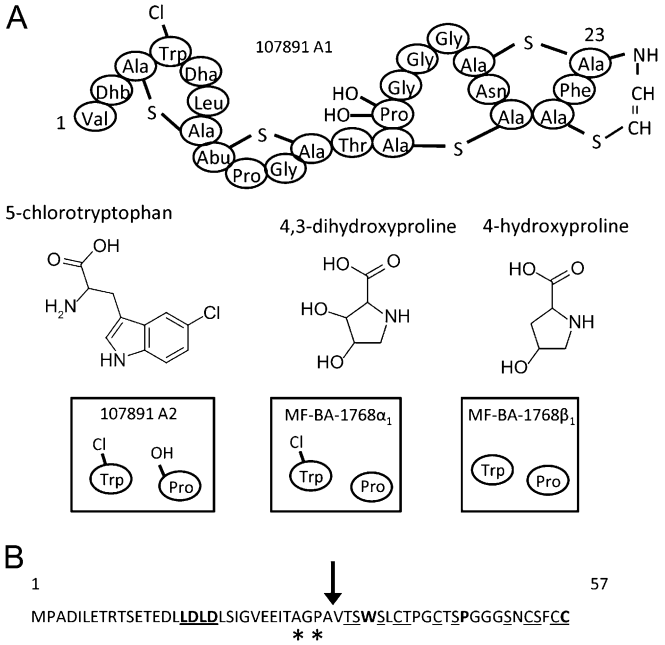


**Fig. 1.** (*A*) Structure of fully modified microbisporicin (107891 A1) (4, 5) and its variants [107891 A2 (4, 5), MF-BA-1768$\alpha_1$, and MF-BA-1768$\beta_1$ (3)]. Structures of 5-chlorotryptophan, 3, 4-dihydroxyproline, and 4-hydroxyproline are shown. Dha, 2, 3-didehydroalanine; Dhb, (*Z*)-2,3-didehydrobutyrine; Abu, l-α-aminobutyric acid. For further information, see Table S1. (*B*) Amino acid sequence of the MibA prepropeptide. The putative site of leader peptide cleavage is shown with a black arrow. Sites where lanthionine bridges will form are underlined. The partially conserved "FNLD" motif (1) (LDLD in MibA) is shown in bold and underlined. Two residues near the putative cleavage site that are conserved in type AI lantibiotics (1) are starred. Residues that are additionally modified by chlorination (W), hydroxylation (P), and amino-vinylcysteine formation (C) are highlighted in bold.

and were found to be clustered together by Southern hybridization. Primers LF001F and LF004R were used to amplify an ≈1,250-bp probe from the *lanC-lanD* region that was hybridized to an *Escherichia coli* cosmid library of *M. corallina* gDNA to identify clones containing the microbisporicin gene cluster. Six cosmids were identified, five of which were shown by PCR and restriction mapping to contain all of the previously identified contigs. One of the cosmids, pIJ12125, was sequenced.

**Genes Within the Biosynthetic Cluster.** The 36,668-bp insert of pIJ12125 contains 33 complete ORFs [determined using Artemis (9); Fig. 2 and Table S2], the products of which are described below.

MibA is the 57-aa prepropeptide of microbisporicin, with a leader sequence extending from residues 1 to 33. The propeptide sequence agrees with the published NMR structure of microbisporicin (Fig. 1*B*) (4, 5). Alignment of the putative leader peptide with those of other lantibiotics, such as nisin, gallidermin, and epidermin, revealed a possibly conserved "FNLD" motif (LDLD in microbisporicin; Fig. 1*B*) potentially involved in processing (10). The MibA cleavage site resembles those of other type AI lantibiotics (Fig. 1*B*), with proline at the −2 position and alanine at −4 (1). *mibA* seems to be at the start of an operon that contains modification and export genes. A 247-bp gap lies between the 5′ end of *mibA* and the preceding oppositely transcribed gene *mibX* and presumably contains the *mibA* promoter, whereas 117 bp of noncoding sequence between *mibA* and *mibB* might reflect the presence of a transcriptional attenuator. Indeed, an RNA secondary structure ($\Delta G = -123$ kJ mol$^{-1}$) was predicted for region 8,820–8,854 bp using Clone Manager 6 (Scientific & Educational Software; Fig. 2). Such structures occur in other lantibiotic gene clusters and are thought to maintain an appropriate stoichiometry between the prepropeptide and the modification enzymes (11).

MibB is a homolog of lantibiotic dehydratases (LanBs). Closest relatives are LanB-like proteins from *Bacillus clausii*, as well as other actinomycetes, such as *Streptomyces noursei* and *Streptomyces griseus* (both with 34% identity), that are not known to make lantibiotics. Homologs from well-studied lantibiotics include SpaB (subtilin), EpiB (epidermin), and MutB (mutacin III). MibB is likely to dehydrate serine and threonine residues in the prepropeptide to yield didehydroalanine and didehydrobutyrine, respectively. MibC shows homology to lantibiotic cyclases (LanCs), including SpaC (subtilin), EpiC (epidermin), and MutC (mutacin III), and is likely to be involved in forming the (methyl-)lanthionine bridges of microbisporicin. MibD is homologous to flavoproteins, specifically those involved in *S*-[(*Z*)-2-aminovinyl]-D-cysteine formation, such as MrsD (mersacidin) and MutD (mutacin III). LanDs decarboxylate the C-terminal cysteine of the peptide chain to yield a free thiol group that subsequently forms a lanthionine bridge via cyclization with a propeptide dehydrated serine or threonine, a reaction likely catalyzed by LanC (1). MibD is presumably involved in formation of the *S*-[(*Z*)-2-aminovinyl]-D-cysteine moiety at the C terminus of microbisporicin (4, 5).

MibT belongs to the ATP-binding cassette (ABC) family of proteins, whereas MibU is homologous to ABC-transporter permeases. MibTU are close homologs of a two-component ABC transporter, CinTH, likely involved in cinnamycin export (7), and probably play a similar role in microbisporicin secretion. MibV is a hypothetical protein with few homologs, the closest

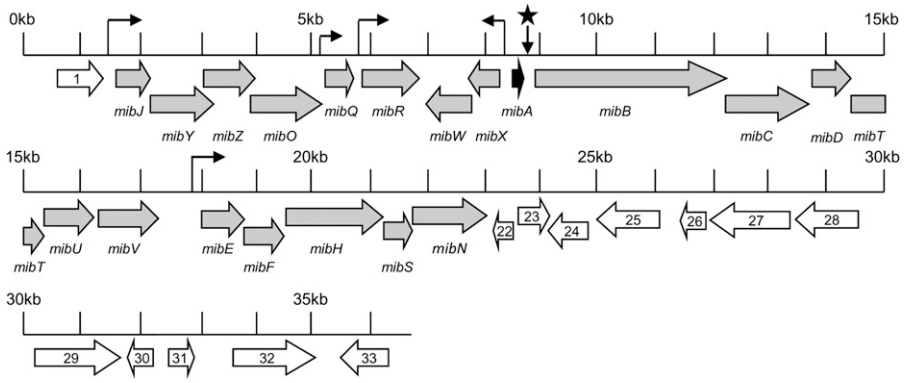


**Fig. 2.** Microbisporicin gene cluster. Each gene is shown to scale, with its position in pIJ12125 marked in kb. *mib* genes are shown in gray. The prepropeptide encoded by *mibA* is shown in black. Other genes on the cosmid are shown in white and are numbered. Black star denotes the position of predicted RNA secondary structure. Line arrows above the scale bar indicate approximate positions of the predicted MibX consensus motif described in the text.

(36% identity) occurring in the actinomycete *Nocardiopsis dassonvillei* and apparently part of an uncharacterized lantibiotic gene cluster. MibE shows homology to ABC-transporter permeases, whereas MibF shares homology with the ABC family of proteins. On the basis of similarity to immunity proteins encoded by other lantibiotic gene clusters [e.g., 24% and 41% amino acid sequence identity, respectively, to LanE and LanF involved in subtilin biosynthesis; permease components typically share lower homology (21–26% identity), likely reflecting their different substrates], MibEF presumably confer producer immunity by actively transporting microbisporicin away from its lipid II target in the membrane (12).

MibH and MibS are homologous to flavin-dependent tryptophan halogenases and flavin reductases, respectively. Flavin-dependent halogenases require a flavin reductase to provide the $FADH_2$ required for chlorination (13). MibH and MibS likely act coordinately to chlorinate tryptophan at position 4 of microbisporicin. MibN shows homology to sodium/proton antiporters, particularly those associated with nonribosomal peptide synthetase and glycopeptide biosynthetic gene clusters, such as ComF (complestatin; *Streptomyces lavendulae*), ForY (fortimicin; *Micromonospora olivasterospora*), NapR2 (napyradiomycin; *Streptomyces aculeolatus*), and StaN (A47934; *Streptomyces toyocaensis*). The role of this family of proteins is not known. Genes downstream of *mibN* seem to be involved in sugar metabolism and are unlikely to be part of the *mib* cluster.

*mibJ*, *mibY*, and *mibZ* seem to be translationally coupled and encode proteins with most homology to the products of a gene cluster in *Frankia* EAN1pec that includes Franean1_3991, a homolog of *mibX*. MibQ shows similarity to lipoproteins, with a predicted signal peptide sequence containing a conserved lipobox motif LAGC (14). MibO is a cytochrome P450 homolog and contains the conserved FGHGxHxCLG motif required for binding heme (15), and it may be responsible for hydroxylation of proline-14 of microbisporicin.

MibR, MibW, and MibX all seem to be regulatory proteins. MibR possesses a helix-turn-helix domain found in the LuxR family of regulatory proteins. MibX belongs to the extracytoplasmic function (ECF) family of σ factors, sharing 49% and 43% amino acid identity with putative ECF σ factors from *Streptosporangium roseum* DSM43021 (Sros_1172) and *Frankia* sp. EAN1pec (Franean1_3991), respectively. Additionally, MibX shares 27% identity with CnrH, an ECF σ factor regulating cobalt and nickel resistance in *Cupriavidus metallidurans* CH34 (previously *Ralstonia metallidurans* CH34) (16). ECF σ factors are a subgroup of RNA polymerase σ factors that respond to extracellular signals (e.g., membrane stress) by directing RNA polymerase to particular promoter sequences. *mibW*, downstream of and apparently translationally coupled to *mibX*, encodes a protein with low levels of similarity to putative anti-σ factors believed to interact with homologs of MibX; MibW homologs occur in *S. roseum* DSM43021 (Sros_1171) and *Frankia* sp. EAN1pec (Franean1_3992), sharing 20% and 18% identity with MibW, respectively. MibW is predicted to have six transmembrane helices and to be embedded in the membrane, with a 73-aa cytoplasmic N-terminal region [TMHMM v.2 (17)]. This, as well as the proximity of *mibW* to *mibX*, suggests that MibW acts as an anti-σ factor to suppress MibX activity.

**Heterologous Expression of the *mib* Gene Cluster.** An ≈5-kb *Ssp*I fragment of pIJ10702 (18), containing the apramycin resistance gene, the ΦC31 integrase gene and corresponding *attB* site, and an *E. coli* origin of transfer was used to replace the kanamycin resistance gene of the SuperCos vector backbone of pIJ12125 by PCR-targeting (19), yielding pIJ12131. Transfer of pIJ12131 to several *Streptomyces* species failed to result in microbisporicin production. *Nonomuraea* sp. ATCC 39727 belongs to the same family as *Microbispora*, the *Streptosporangiaceae*, and produces the glycopeptide antibiotic A40926 (20). pIJ12131 was transferred into *Nonomuraea* sp. ATCC 39727 by conjugation from *E. coli* ET12567 pUZ8002 and integration at the ΦC31 attachment site (21) confirmed by PCR. Whereas the culture supernatant from a representative exconjugant clearly inhibited growth of *Micrococcus luteus* (Fig. 3), those from wild-type *Nonomuraea* and a vector-only control did not. Production of microbisporicin was confirmed by MALDI-TOF analysis of the same culture supernatant (Fig. S1). Thus, all of the genes required for microbisporicin production lie within the 36,668-bp insert of pIJ12131.

**Generation of a *mibA* Deletion Mutant.** A method for the genetic manipulation of *M. corallina* was developed (*SI Results*; Fig. S2). *mibA* of pIJ12125 (which confers kanamycin resistance) was replaced with an apramycin resistance cassette [*aac(3)IV*] by PCR targeting. The resulting construct was introduced into *M. corallina* by conjugation from ET12567 pUZ8002 and exconjugants selected using apramycin. Double-crossover recombinants in which *mibA* was replaced by the mutant allele were identified by their apramycin-resistant and kanamycin-sensitive phenotypes and confirmed as Δ*mibA*::*aac(3)IV* by Southern hybridization using pIJ12125 as probe. *M. corallina* Δ*mibA*::*aac(3)IV* and the parental strain were grown for 7 d in liquid culture. In contrast to the wild-type strain, culture supernatant from the Δ*mibA* mutant failed to inhibit *M. luteus* (Fig. 4), and no microbisporicin was detected by MALDI-TOF analysis (Fig. S3). Thus, *mibA* is essential for microbisporicin biosynthesis in *M. corallina*, confirming the identity of the gene cluster.

**Deletion Analysis.** Deletion mutants were similarly generated in *M. corallina* for *mibD*, *mibTU*, *mibEF*, *mibN*, *mibH*, *mibV*, and *mibX*. An ≈7-kb segment downstream of *mibN* was also replaced with the apramycin cassette. Mutations were confirmed by PCR and Southern hybridization. Unless stated otherwise, at least two independent clones for each mutant were analyzed and compared with the wild-type strain using bioassays against *M. luteus* (Fig. 4) and MALDI-TOF mass spectrometry (Fig. S3) of supernatants from 7-d cultures.

Deletion of the 7-kb segment had no effect on microbisporicin production, thus defining the right-hand boundary of the biosynthetic gene cluster. Deletion of *mibD* resulted in loss of bioactivity and disappearance of MALDI-TOF peaks associated with the microbisporicin complex. Microbisporicin production was restored to wild-type levels by the *in trans* expression of *mibD* from the *mibA* promoter (likely to be its native promoter). Surprisingly, deletion of *mibTU* (only one clone tested) had no effect on microbisporicin production when assessed by both bioassay and

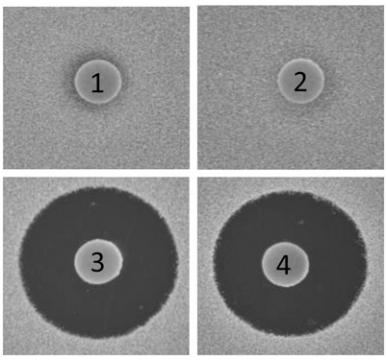


**Fig. 3.** Heterologous production of microbisporicin in *Nonomuraea sp.* ATCC 39727. *Nonomuraea sp.* ATCC 39727 wild type (1), containing pIJ10702 vector alone (2), containing pIJ12131 (3), and *M. corallina* NRRL 30420 (4) were grown for 7 d in VSP medium and 40 μL of culture supernatant assayed for activity against *M. luteus*.

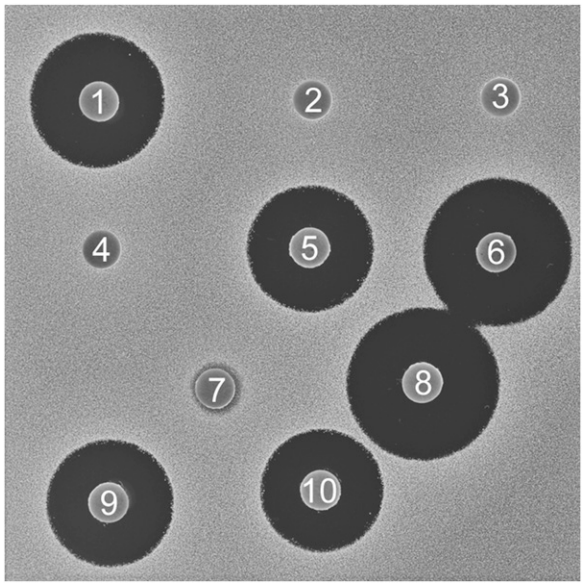


**Fig. 4.** Analysis of bioactivity from deletion mutants of *M. corallina* NRRL 30420. *M. corallina* NRRL 30420 wild type (1), Δ*mibA::aac(3)IV* (2), Δ*mibX::aac(3)IV* (3), Δ*mibD::aac(3)IV* (4), Δ*mibTU::aac(3)IV* (5), Δ*mibV::aac(3)IV* (6), Δ*mibEF::aac(3)IV* (7), Δ*mibH::aac(3)IV* (8), Δ*mibN::aac(3)IV* (9), and Δdownstream::*aac(3)IV* (10) were grown for 7 d in VSP medium and culture supernatants assayed for bioactivity as in Fig. 3.

MALDI-TOF mass spectrometry. Presumably there are other ABC transporters in *M. corallina*, either in the *mib* cluster or elsewhere in the genome, able to export the lantibiotic. Deletion of *mibN* also had no apparent effect on microbisporicin production. In contrast, deletion of *mibEF* resulted in markedly delayed and greatly reduced levels of microbisporicin production. Whereas supernatants from 3- to 5-d cultures failed (unlike the wild type) to inhibit *M. luteus*, those from 7-d cultures gave a small zone of inhibition. Mass spectrometry revealed small amounts of the microbisporicin complex in the 7-d culture supernatants. Thus, *mibEF* are essential for wild-type levels of microbisporicin production, which were restored by the *in trans* expression of *mibEF* from their native promoter.

Culture supernatants of the *mibH* mutant retained bioactivity, but MALDI-TOF analysis revealed *m/z* peaks attributable only to the nonchlorinated form of microbisporicin (Table S1) (3). The presence of deschloromicrobisporicin in these samples is confirmed by the shift in the observed isotope pattern (Fig. S4). Expression *in trans* of *mibHS* from the *mibEF* promoter (likely to be the native promoter) revealed masses attributable to both the chlorinated and nonchlorinated forms of microbisporicin. Thus, MibH seems to be the halogenase responsible for chlorination of microbisporicin. Deletion of *mibV* yielded essentially the same results. However, MALDI-TOF analysis of culture supernatants of three independent *mibV* mutants revealed additional compounds with masses lower than those of BA-1768$\beta_1$ that may be microbisporicin degradation products.

Deletion of *mibX* (only one clone tested) resulted in loss of microbisporicin production, assessed by activity of culture supernatants against *M. luteus* and MALDI-TOF mass spectrometry. The *in trans* expression of *mibX* from its native promoter restored microbisporicin production.

**Regulation of Microbisporicin Production by MibX.** Microbisporicin (detected by activity against *M. luteus*) was first observed after 46–50 h of growth in medium V supplemented with sucrose and proline (VSP medium), corresponding to midexponential growth phase (Fig. S5*A*). To assess the potential regulatory role of *mibX*, RNA was isolated after 48 h and 72 h from the wild-type strain and from the *mibA* and *mibX* mutants (Fig. S5*B*) and analyzed by RT-PCR. To provide an internal standard, we identified a homolog of the major vegetative σ factor gene, *hrdB*, of *Streptomyces coelicolor* (SCO5820), in the *M. corallina* 454 sequence data (91% nucleotide identity across 509 nucleotides of C-terminal sequence). *M. corallina hrdB* was expressed at roughly equal levels in all three strains (two biological replicates for each strain at both time points) at both 48 h and 72 h of growth (Fig. 5 and Fig. S6*A*).

Expression analysis was carried out using primers listed in Table S3. Of the genes monitored, only *orf1* was expressed at approximately equivalent levels in all three strains at both time points; *orf1* lies at one end of the cloned *M. corallina* DNA and does not seem to be cotranscribed with other putative *mib* genes. Transcription of all of the other genes was reduced in both the *mibA* and *mibX* mutants compared with the wild-type strain at both 48 h and 72 h of cultivation (Fig. 5 and Fig. S6*A*). The *mibA* transcript was absent in the *mibX* mutant, and the *mibX* transcript was markedly reduced in the *mibA* mutant. Thus, both *mibA* and *mibX* are required for wild-type expression of the *mib* genes. The expression patterns of *mibYOQRWX* (Fig. S6*A*) indicated that they were also likely to be involved in microbisporicin biosynthesis.

We next analyzed all of the putative intergenic regions (*orf* 1-*mibJ*, *mibO-mibQ*, *mibQ-mibR*, *mibX-mibA*, and *mibV-mibE*) for a consensus sequence using MEME (22). This revealed a consensus motif of GAACC-N15-GCTAC for all five sequences (Fig. 2 and Fig. S6). Only the transcription unit starting with *mibA* lacked the motif. The consensus sequence strongly resembles those of well-characterized ECF σ factors (AAC in the −35 region and CGT in the −10 region) (23). Together, these results suggest that all of the *mib* transcription units are coregulated by the ECF σ factor MibX.

## Discussion

Microbisporicin is a highly promising candidate antibiotic for clinical development (4, 5) and as a gene-encoded peptide has significant potential for rational design (1, 6). The identification and characterization of the genes responsible for microbisporicin biosynthesis, and the development of methods for the genetic manipulation of the producing organism, provide the foundation for knowledge-based increases in strain productivity and the generation of variants with potentially improved pharmacological properties. Consequently, the work described here may represent a key milestone in the future clinical use of this potent antibiotic.

This study reports the genetic manipulation of a *Microbispora* strain and analysis of a type AI lantibiotic gene cluster from an actinomycete. Moreover, deletion analysis has identified functions for several *mib* genes.

Microbisporicin is unique among lantibiotics and other ribosomally synthesized peptides in containing a chlorinated tryptophan, and we have identified a flavin-dependent tryptophan halogenase involved in modifying such peptide substrates. MibH shares relatively low amino acid sequence identity with other flavin-dependent halogenases, for example, 24% and 30% with PrnA and PyrH involved in pyrollnitrin [*Pseudomonas fluorescens* (13)] and pyrroindomycin [*Streptomyces rugosporus* (8)] production, respectively, whereas the latter two enzymes share 41% identity. This might reflect differences in the type of substrate; whereas PrnA and PyrH act on free tryptophan (8, 13), MibH presumably acts post-translationally on a potentially structured peptide. Future studies of MibH may reveal the structural and sequence differences that discriminate between the two types of substrate and determine how readily MibH can be used to chlorinate other peptides.

Deletion of *mibV* also resulted in the production of nonchlorinated microbisporicin. The presence of potential degradation

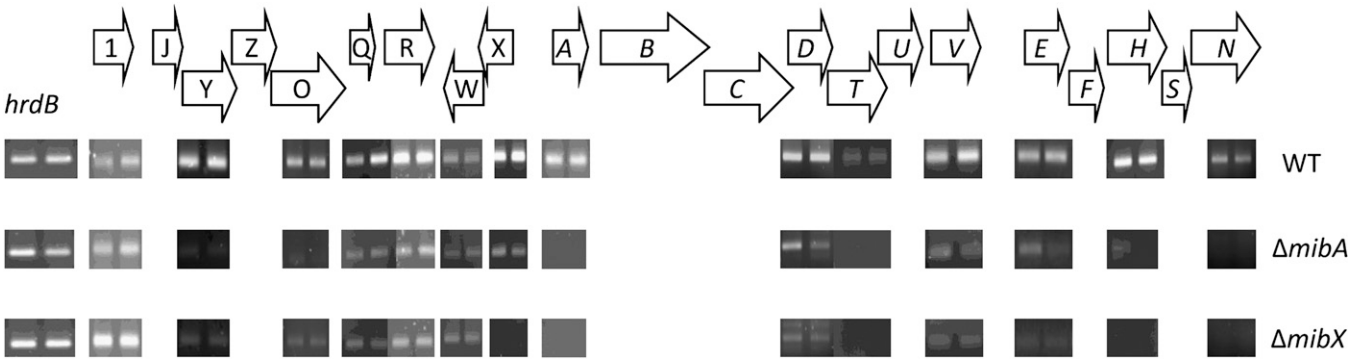


**Fig. 5.** RT-PCR analysis of *mib* gene expression during midexponential growth (48 h) of *M. corallina* wild type (WT), Δ*mibA::aac(3)IV* (Δ*mibA*), and Δ*mibX::aac(3)IV* (Δ*mibX*). Two samples of RNA were isolated from each strain (shown side by side for each primer set) and used for RT-PCR (primer pairs are listed in Table S3). Primers internal to *hrdB* of *M. corallina* were used as a control (*Left*).

products in the *mibV* mutant might reflect a role for MibV during peptide maturation, perhaps acting as a scaffold or chaperone. Interaction between MibV and MibH might then be essential for chlorination to occur, explaining the similar mutant phenotypes. *mibH* and *mibV* mutants reproducibly exhibited higher than wild-type levels of activity against *M. luteus* (Fig. 4), suggesting that the nonchlorinated form of microbisporicin might be more active. Although there are conflicting reports about the relative activities of the chlorinated and nonchlorinated forms of the lantibiotic (3–5), its unusual activity against some Gram-negative pathogens might reflect increased penetrability, potentially attributable to the increase in net charge that results from chlorination.

Hydroxylation of proline-14 of microbisporicin is another unique lantibiotic modification. Cytochrome P450s catalyze the regiospecific and stereospecific oxidation of nonactivated hydrocarbons, frequently acting as tailoring enzymes in the biosynthesis of polyketides and non–ribosomally synthesized peptides (24). Thus, it seems likely that MibO is responsible for the unique dihydroxylation of proline-14 of microbisporicin.

The absence of microbisporicin variants in the culture supernatant of the *mibD* mutant lacking the C-terminal *S*-[(*Z*)-2-aminovinyl]-D-cysteine suggests either that this modification is essential for full maturation or that the peptide is rapidly degraded in its absence. Interestingly, many lantibiotics possess (methyl-)lanthionine bridges or *S*-[(*Z*)-2-aminovinyl]-D-cysteines at their C termini (1) that might serve to prevent carboxypeptidase degradation in the external environment.

On the basis of homology, MibEF are likely to confer immunity to microbisporicin. Deletion of *mibEF* resulted in very low levels of bioactivity late in growth, potentially reflecting release of the compound by cell lysis in stationary phase. In the absence of MibEF-mediated immunity, the cell may respond by blocking microbisporcin export or even markedly reducing *mib* gene expression. Alternatively, despite the presence of MibTU, MibEF might be solely responsible for export of microbisporicin.

An enzyme responsible for leader peptide cleavage was not identified in the *mib* cluster. Although many lantibiotic gene clusters encode dedicated extracellular proteases (LanP) that fulfill this role (1, 25), some do not and seem to rely on nonspecific proteases for leader peptide cleavage (1, 7). Such proteases are involved in maturation of haloduracin (26). This may also be the case for microbisporicin; production of the mature peptide in *Nonomuraea* is also consistent with a role for a general protease in leader peptide cleavage.

The ECF σ factor family, to which MibX belongs, was discovered in *S. coelicolor* ($\sigma^E$), but members have since been identified in a wide variety of bacteria. Several mechanisms regulate σ factor activity (27), including control by anti-σ factors that sequester the σ factor, thus preventing transcription initiation from cognate promoters until receipt of a specific signal. The putative anti-σ factor MibW is predicted to be located in the membrane but with a cytoplasmic N-terminal segment that could interact with and sequester the MibX ECF σ factor. Indeed, bacterial two-hybrid analysis in *E. coli* demonstrated strong interaction between MibX and MibW. We suggest a model in which MibX is required for high-level expression of the microbisporicin gene cluster (including its own gene) and in which its activity depends on low levels of production of microbisporicin. We propose that low levels of microbisporicin (potentially produced by expression from a starvation-induced promoter) or cell envelope stress (induced by the likely interaction of microbisporicin with lipid II) prevents MibW from interacting with MibX, thus releasing the ECF σ factor and resulting in high-level expression of the entire microbisporicin gene cluster. Interestingly, the operon beginning with *mibA* does not have the consensus motif, suggesting that regulation by MibX might be mediated through a gene in one of the other MibX-regulated operons. A likely candidate for this is *mibR*, which encodes a protein with a helix-turn-helix DNA binding domain.

Until now, ECF σ factors were associated almost exclusively with mediating global or pleiotropic changes in gene expression in response to cell stress or during development (23). Very few examples of pathway-specific ECF σ factors have been reported and to our knowledge none that specifically regulate antibiotic production. Biosynthesis of sublancin, a type A(II) lantibiotic produced by *Bacillus subtilis* strains that are lysogenic for the SPβ bacteriophage, is dependent on $\sigma^X$ and, to a lesser extent, $\sigma^M$. However, this effect is mediated indirectly through the transition state regulator Abh (28). The microbisporcin gene cluster provides an example of the involvement of an ECF σ factor as a pathway-specific regulator of antibiotic biosynthesis.

## Experimental Procedures

For detailed information, see *SI Experimental Procedures*.

**Strains and General Methods.** Oligonucleotides and constructs are described in Tables S3 and S4. *M. corallina* (NRRL 30420) was grown on V0.1 solid medium or in VSP liquid medium (29) (*SI Experimental Procedures*). *Nonomuraea* sp. ATCC 39727 was a kind gift from Professor Flavia Marinelli (Università dell'Insubria, Varese, Italy) and was grown on V0.1 or in VSP (29) (*SI Experimental Procedures*). Conjugations into *Nonomuraea* were carried out as in ref. 21. *Streptomyces* strains were cultured and manipulated as in ref. 30. *E. coli* was cultured according to (31). Southern analysis was carried out as described in ref. 31 and *SI Experimental Procedures*.

**Identification of the *mib* Gene Cluster.** Genomic DNA from *M. corallina* mycelium was subjected to 454 sequencing (Cogenics). The resulting 3.34 Mb of sequence (with 3,027 contigs >500 nt and mean size 1,117 bp) was interrogated for protein and nucleotide sequences of interest using BlastN and tBlastN, respectively (32). Searches were carried out using the amino acid sequences of

lantibiotic biosynthetic enzymes, the presumed unmodified propeptide of microbisporicin (VTSWSLCTPGCTSPGGGSNCSFCC), and several flavin adenine dinucleotide (FAD)-dependent tryptophan halogenases. A cosmid library of *M. corallina* gNDA was constructed and screened for cosmids hybridizing to a 1,250-bp probe spanning *mibC-mibD*. The sequence of pIJ12125 was determined by Sanger sequencing (Cambridge University DNA Sequencing Service) and annotated using Artemis (Sanger Institute) (9). ORFs were further characterized by homology searches using National Center for Biotechnology Information Blast.

**Heterologous Expression.** pIJ12125 was targeted with a 5,247-bp *Ssp*I fragment of pIJ10702 (18) as described in ref. 19. The resulting cosmid, pIJ12131, was confirmed by restriction digestion, transferred to *E. coli* ET12567 pUZ8002 by transformation, and thence into the heterologous host by conjugation. Exconjugants were selected with 50 μg/mL apramycin.

**Detection of Microbisporicin.** Forty-microliter samples of culture supernatants and controls were applied to antibiotic assay discs, allowed to dry, and placed onto lawns of *M. luteus* that were incubated at 30 °C until halos were visible, usually overnight. MALDI-TOF mass spectrometry was carried out as described in *SI Experimental Procedures*.

**Manipulation of *M. corallina*.** Conjugation between *E. coli* ET12567 pUZ8002 carrying the *oriT*-containing plasmid and *M. corallina* was carried out as described in *SI Experimental Procedures*. To generate deletion mutants, genes in pIJ12125 were replaced with an apramycin$^R$-oriT cassette amplified from pIJ773 using the primer pairs listed in Table S3, as described in ref. 19. Mutations were confirmed by PCR using flanking primers and by restriction digests to confirm the integrity of the cosmid. Mutant cosmids were transferred to *E. coli* ET12567 pUZ8002 by transformation (31) and the resulting strain conjugated with *M. corallina* mycelium. Ex-conjugants were selected on V0.1 containing 50 μg/mL apramycin and subcultured in VSP containing 25 μg/mL apramycin for 6 d at 30 °C before streaking for single colonies on V0.1 containing 50 μg/mL apramycin. Single colonies were assayed for growth on V0.1 containing 50 μg/mL kanamycin. Clones sensitive to kanamycin were cultured in VSP containing 50 μg/mL apramycin for 6 d at 30 °C and stored as a master cell bank at −80 °C. A further subculture was grown in VSP containing 25 μg/mL apramycin at 30 °C for 48 h to prepare gDNA, which was analyzed by PCR. Genotypes of clones were further confirmed by Southern hybridization. Complementation was carried out *in trans* by expressing a wild-type copy of the gene from its native promoter (*SI Experimental Procedures*).

**RNA Preparation from *M. corallina* and RT-PCR.** RNA was prepared from *M. corallina* mycelium grown in liquid VSP medium. Mycelium was lysed in a FastPrep machine (Thermo Scientific) at speed 6.5 for 30 s and RNA purified using the RNeasy Mini Kit (Qiagen) as described in *SI Experimental Procedures*. RNA concentration and quality were assessed and RT-PCR performed as described in *SI Experimental Procedures*.

**ACKNOWLEDGMENTS.** We thank David Hopwood for comments on the manuscript; Govind Chandra for assistance with bioinformatics; Giorgia Letizia Marcone, Flavia Marinelli, and Fabrizio Beltrametti for advice on culture conditions for *Nonomuraea* and *Microbispora*; Sean O'Rourke (John Innes Centre, Norwich, UK) for the gift of pIJ10706; Andy Hesketh and Sean O'Rourke for technical advice; and Gerhard Saalbach and Mike Naldrett for mass spectrometry. This work was supported financially by a Doctoral Training Grant to L.C.F. and by funding to M.J.B., both from the Biotechnology and Biological Sciences Research Council, United Kingdom.

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