Cloning and Analysis of the Planosporicin Lantibiotic Biosynthetic Gene Cluster of *Planomonospora alba*

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The increasing prevalence of antibiotic resistance in bacterial pathogens has renewed focus on natural products with antimicrobial properties. Lantibiotics are ribosomally synthesized peptide antibiotics that are posttranslationally modified to introduce (methyl)lanthionine bridges. Actinomycetes are renowned for their ability to produce a large variety of antibiotics, many with clinical applications, but are known to make only a few lantibiotics. One such compound is planosporicin produced by *Planomonospora alba*, which inhibits cell wall biosynthesis in Gram-positive pathogens. Planosporicin is a type AI lantibiotic structurally similar to those which bind lipid II, the immediate precursor for cell wall biosynthesis. The gene cluster responsible for planosporicin biosynthesis was identified by genome mining and subsequently isolated from a *P. alba* cosmid library. A minimal cluster of 15 genes sufficient for planosporicin production was defined by heterologous expression in *Nonomuraea* sp. strain ATCC 39727, while deletion of the gene encoding the precursor peptide from *P. alba*, which abolished planosporicin production, was also used to confirm the identity of the gene cluster. Deletion of genes encoding likely biosynthetic enzymes identified through bioinformatic analysis revealed that they, too, are essential for planosporicin production in the native host. Reverse transcription-PCR (RT-PCR) analysis indicated that the planosporicin gene cluster is transcribed in three operons. Expression of one of these, *pspEF*, which encodes an ABC transporter, in *Streptomyces coelicolor* A3(2) conferred some degree of planosporicin resistance on the heterologous host. The inability to delete these genes from *P. alba* suggests that they play an essential role in immunity in the natural producer.

More than two-thirds of clinically used antibiotics are natural products or their derivatives (1). These naturally derived compounds possess structural complexity and bioactivity that are difficult to achieve by chemical synthesis, and the failure of high-throughput target-based screening of chemical libraries to deliver clinically useful antibiotics has prompted renewed interest in natural products with antimicrobial activity. Lanthipeptides are a class of ribosomally synthesized and posttranslationally modified peptides (RiPPs) that have been particularly well studied. Lanthipeptides are characterized by the presence of the ring-forming thioether amino acids lanthionine (Lan) and/or 3-methyl-lanthionine (MeLan) (Fig. 1A) (2). These nonproteogenic amino acids constrain the molecule into a defined structural conformation that serves the dual role of promoting target specificity while resisting proteolytic degradation (5). Lanthipeptides are subdivided into four different classes (I to IV) depending on the biosynthetic enzymes that install the Lan and MeLan motifs (6). Their origin from a gene-encoded precursor peptide, together with recent advances in DNA sequencing, has allowed genome mining to reveal several new classes of RiPPs, many of which contain novel post-translational modifications (7), and has led to renewed interest in this fascinating group of molecules.

Lanthipeptides that exhibit antimicrobial activity are referred to as lantibiotics. The first characterized lantibiotic, nisin, was discovered over 80 years ago (8), yet the ribosomal origin of such modified peptides was only revealed 60 years later when the epidermin biosynthetic gene cluster of *Staphylococcus epidermidis* Tü 3298 was sequenced (9). Since then, nearly 100 lantibiotics have been characterized, most of which are produced by low-GC Gram-positive bacteria.

Actinobacteria are prolific producers of natural products and account for more than two-thirds of all naturally occurring antibiotics (10). Although not thought of as a major source of RiPPs,

a number of actinomycete lanthipeptide biosynthetic gene clusters have been characterized, including those for cinnamycin from *Streptomyces cinnamoneus* subsp. *cinnamoneus* DSM 40005 (11), SapB from *Streptomyces coelicolor* (12), SapT from *Streptomyces tendae* (13), actagardine from *Actinoplanes garbadinensis* (14), deoxyactagardine B (DAB) from *Actinoplanes liguriae* (15), venezuelin from *Streptomyces venezuelae* (16), and microbisporicin from *Microbispora corallina* (17).

Several of these actinomycete lanthipeptides are of clinical interest. Recently, NVB302 (an actagardine derivative) and Moli1901 (also known as lancovutide or duramycin, a structural analogue of cinnamycin) successfully completed phase I and phase II clinical trials for the treatment of *Clostridium difficile* infections (18) and cystic fibrosis (19, 20), respectively. In addition, NAI-107 (also known as microbisporicin) is in late-stage preclinical development for the treatment of multidrug-resistant Gram-positive pathogens (21).

Planosporicin is another actinomycete-derived lantibiotic produced by *Planomonospora* sp. strain DSM 14920 (22). While

Received 31 December 2012 Accepted 4 March 2013

Published ahead of print 8 March 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.02291-12>.

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doi:10.1128/JB.02291-12

FIG 1 (A) Structural motifs found in planosporicin and microbisporicin introduced through posttranslational modifications. Orange circles represent the peptide backbone. The notation used to represent these modifications in panel B is also shown. (B) Structures of planosporicin (3) and microbisporicin (4). Residues are unmodified (white), dehydrated (purple), dehydrated and cyclized (blue), or modified in other ways (green). Abbreviations: Abu, aminobutyric acid; Dha, dehydroalanine; Ala-S-Ala, lanthionine; Dhb, dehydrobutyrine; Abu-S-Ala, methyl-lanthionine. N-terminal A and B rings are in gray.

thought originally to possess a novel globular form (22), subsequent studies revealed an elongated structure with one methyl-lanthionine and four lanthionine bridges (Fig. 1B) (3) and suggested that planosporicin was indeed ribosomally synthesized. Evidence thus far indicates that planosporicin inhibits cell wall biosynthesis (22), and its N-terminal similarity to nisin (23) and microbisporicin (4) suggests an ability to bind to lipid II, the immediate precursor for cell wall biosynthesis (24). *Planomonospora* is a genus in the family *Streptosporangiaceae* with a high genomic GC content that forms a highly differentiated, branched mycelium that sporulates to form clavate monospores (25). Screening of *Planomonospora* species from publicly available culture collections revealed that one of them, *Planomonospora alba*, also produced planosporicin. Here we report the identification, cloning, and analysis of the planosporicin biosynthetic gene cluster of *P. alba*.

MATERIALS AND METHODS

Strains and general methods. Strains and plasmids are described in Table 1 and in Table S1 in the supplemental material, respectively. All *Planomonospora* strains were obtained from the ARS Culture Collection, Peoria, IL, and grown using ISP4 (29) for the generation of biomass and AF/MS (dextrose, 20 g/liter; yeast extract, 2 g/liter; organic soy flour, 8 g/liter; NaCl, 1 g/liter; and CaCO₃, 4 g/liter, with the pH adjusted to 7.3) (22) for the production of planosporicin (see the supplemental material). *Nonomuraea* sp. strain ATCC 39727 was a gift from Flavia Marinelli (Università del Insubria, Varese, Italy) and was cultured and manipulated as described in reference 17. *Streptomyces* and *Escherichia coli* strains were cultured and manipulated as described in references 30 and 31, respectively. DNA oligonucleotides were purchased from Sigma-Aldrich and are listed in Table S2 in the supplemental material. Restriction and PCR enzymes were purchased from Roche or New England BioLabs. DNA fragments were generally cloned through amplification using the Expand high-fidelity PCR system (Roche) and TA cloned into pGEM-T (Promega) for sequencing (The Genome Analysis Centre, Norwich Research Park). Error-free fragments were subsequently excised and ligated into the

relevant vector. All subcloning was carried out in *E. coli* DH5α (Invitrogen).

Identification of the *psp* gene cluster. High-molecular-weight genomic DNA (gDNA) isolated from *P. alba* (see the supplemental material) was sequenced using 454 sequencing technology (The Genome Analysis Centre, Norwich Research Park). One-quarter of a run of 454 sequencing yielded 73 Mb of sequence data which were assembled using Newbler assembly software to give 944 contigs with a mean length of 7,718 bp. The assembled contig sequences were used to construct a database using the format for the BLAST suite of programs, version 2.2.18 (National Center for Biotechnology Information [NCBI] [32]). TblastN searches using protein sequences of interest, including the presumed unmodified precursor peptide of planosporicin (ITSVSWCTPGCTSEGGG SGCSHSS), revealed contigs likely to contain part of the planosporicin biosynthetic gene cluster.

Three probes were amplified from *P. alba* gDNA by PCR using three pairs of primers designed from 454 sequence data. Primers 1289FlanA and 1289RlanA amplified 230 bp that included the presumptive *pspA*. 3088F and 3088R amplified 547 bp of the putative *pspB*. PMSlanAf and PMSlanBf amplified 1,701 bp spanning the presumptive *pspA* and 5' end of *pspB*. The PCR products were cloned into pGEM-T (Promega), confirmed by sequencing, PCR reamplified from a single clone, and purified (Qiagen).

The three probes were hybridized separately with three copies of the *P. alba* cosmid library (see the supplemental material). Cosmids pIJ12321 and pIJ12322 were sequenced using Sanger sequencing by the University of Cambridge DNA Sequencing Service. The cloned sequences were annotated using Artemis software (33). Protein coding sequences (PCs) were predicted on the basis of the GC content across triplets (34). Likely translational start sites (ATG, GTG, and TTG) were identified by the presence of ribosome-binding sites (RBSs) approximately 6 to 10 nucleotides (nt) upstream of translational start sites and based on the sequence GGAGG. BLASTP searches of the NCBI database using the deduced amino acid sequences were used to annotate the putative PCs.

Heterologous expression. pIJ12321 was PCR targeted to introduce an integrase (*intΦC31*), phage attachment site (*attP*), origin of transfer (*oriT*), and apramycin resistance cassette [*aac(3)IV*], creating pIJ12323.

TABLE 1 Strains used in this study

Strain	Description	Source or reference
<i>S. lividans</i> TK24 and derivatives		
TK24	<i>S. lividans</i> 1326 (SLP2 [−] SLP3 [−] <i>str-6</i>)	26
M1446	TK24 pIJ10702 in Φ C31 <i>attB</i> (empty vector)	Jan Claesen, UCSF
M1286	TK24 pIJ12323 in Φ C31 <i>attB</i> (B4-1 cosmid)	This work
<i>S. coelicolor</i> M1146 and derivatives		
M1146	M145 Δ act Δ red Δ cpk Δ cda	27
M1410	M1146 pIJ10702 in Φ C31 <i>attB</i> (empty vector)	28
M1288	M1146 pIJ12323 in Φ C31 <i>attB</i> (B4-1 cosmid)	This work
<i>Nonomuraea</i> strain ATCC 39727 and derivatives		
ATCC 39727	Wild-type strain	Flavia Marinelli, Università del l'Insubria, Italy
M1294	pIJ10702 in Φ C31 <i>attB</i> (empty vector)	17
M1295	pIJ12323 in Φ C31 <i>attB</i> (B4-1 cosmid)	This work
M1296	pIJ12324 in Φ C31 <i>attB</i> (F13-1 cosmid)	This work
M1299	pIJ12327 in Φ C31 <i>attB</i> (<i>pcs-19</i> to <i>pspV</i>)	This work
M1300	pIJ12328 in Φ C31 <i>attB</i> (<i>pcs-11</i> to <i>pspV</i>)	This work
M1301	pIJ12329 in Φ C31 <i>attB</i> (<i>pspE</i> to <i>pspV</i>)	This work
<i>P. alba</i> and derivatives		
NRRL 18924	Wild-type strain	25
M1302	Δ pspA::(<i>oriT</i> - <i>hyg</i>)	This work
M1303	Δ pspX::(<i>oriT</i> - <i>hyg</i>)	This work
M1304	Δ pspW::(<i>oriT</i> - <i>hyg</i>)	This work
M1305	Δ pspYZ::(<i>oriT</i> - <i>hyg</i>)	This work
M1306	Δ pspTU::(<i>oriT</i> - <i>hyg</i>)	This work
M1307	Δ pspV::(<i>oriT</i> - <i>hyg</i>)	This work
M1308	Δ pspR::(<i>oriT</i> - <i>hyg</i>)	This work
M1309	Δ pspJ::(<i>oriT</i> - <i>hyg</i>)	This work
M1310	Δ pspQ::(<i>oriT</i> - <i>hyg</i>)	This work
<i>S. coelicolor</i> M1152 and derivatives		
M1152	M145 Δ act Δ red Δ cpk Δ cda <i>rpoB</i> [C1298T]	27
M1553	pIJ12717 in Φ BT1 <i>attB</i> (<i>ermE</i> *p- <i>pspEF</i>)	This work

pIJ12322 was similarly targeted to create pIJ12324. This was achieved through the replacement of *neo* on the SuperCosI backbone with the 5,247-bp *oriT-attP-int-aac(3)IV* fragment (flanked by *SspI* sites) from pIJ10702 (also known as pMJCOS1) through double-crossover recombination mediated by sequence homology in the regions flanking both the resident resistance gene and the incoming cassette, as described previously (35). Cosmid DNA that had been successfully targeted with the pIJ10702 *SspI* fragment was confirmed by *NotI* restriction digestion to reveal a diagnostic band shift from 6,807 bp to 8,682 bp. pIJ12323 and pIJ12324 were introduced into *E. coli* ET12567/pUZ8002 by transformation and then conjugated into the heterologous host from which exconjugants were selected with 50 μ g/ml apramycin.

Construction of minimal gene set. A minimal gene set was created by deleting DNA outside the proposed biosynthetic gene cluster. The P1-FRT-apramycin resistance-*oriT*-FRT-P2 cassette from pIJ773 was the template used to amplify the extended resistance cassette. The primers were designed to contain an additional restriction site for an enzyme that did not cut elsewhere on the cosmid. This site is located between the universal primer binding site (P1/P2) and the 39-bp extension homologous to the cosmid. Gene replacement was carried out in *E. coli* BW25113/pIJ790 containing pIJ12321 as described previously (35). The region of the cosmid downstream of the biosynthetic gene cluster was targeted using a cassette amplified using the *downdisruptF* and *downdisruptR* primers, and then a preparative digestion was performed with *SpeI* and the construct religated. The deleted cosmid was confirmed by PCR (primers *downconfirm* and *end_R*), and a diagnostic restriction digest before the

region upstream of the *psp* cluster was targeted as described above. A preparative digestion with *XbaI* and religation of the construct removed the resistance cassette to create the minimal *psp* gene set. This was confirmed by PCR (primers *upconfirm* and *end_F*) and then targeted to replace *neo* on the SuperCosI backbone with the *oriT-attP-int-aac(3)IV* cassette as described above, yielding pIJ12329 (see Table S1 in the supplemental material). At each stage, the cosmid derivatives were confirmed by PCR with primers flanking the deleted region (see Table S2 in the supplemental material), as well as by diagnostic restriction enzyme digests.

Isolation and purification of planosporicin. Both culture supernatants and mycelium were examined for the presence of planosporicin. *P. alba* or *Streptomyces* mycelium was collected by centrifugation. The culture supernatant was passed through 0.2- μ m (Millipore) filters; the mycelium was extracted with MeOH, vortexed, and sonicated (Branston 2510), and 1 ml of the extract was dried down to $\sim$ 100 μ l in a miVac Duo concentrator (GeneVac).

Concentration of planosporicin from the supernatant was carried out using a method based on that described in reference 22. Filtered supernatant was acidified to $\sim$ pH 3 with concentrated sulfuric acid. Diaion HP20 polystyrene resin (Mitsubishi Chemical Co.) was prepared by washing in 100% methanol and then rinsed in water before being added to the supernatant at 2.6% (vol/vol) and incubated for 2 h at 4°C on a vertical rotating mixer. The resin was recovered by centrifugation, washed in 5 ml methanol-water (1:1 [vol/vol]) for 1 min, and eluted in 1 ml methanol-water-saturated butanol-water (9:1:1 [vol/vol/vol]) for 5 min; this procedure was repeated to give a series of fractions. The spent supernatant, wash, and

FIG 2 Precursor ion scan on concentrated *P. alba* culture supernatant. Eighty ESI scans were combined and processed. Each peak represents the doubly charged ion $[M + 2H]^{2+}$, with m/z labeled above. The y axis shows the intensity normalized to the highest peak, and m/z is displayed on the x axis. The parent ion (1,096.9027 Da) is indicated by an arrow.

eluent were stored for later analysis. Samples were either assayed directly for bioactivity or further concentrated by drying in a miVac Duo concentrator (GeneVac).

Detection of planosporicin by bioassay. *Micrococcus luteus* was grown in L broth at 30°C with shaking to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6. The culture was diluted 1 in 10 either into molten soft nutrient agar to overlay agar plates or into molten L agar and poured into 100-mm square petri dishes to assay liquid samples. Supernatants or extracts (two 20- μ l samples) were spotted onto sterile antibiotic assay discs which were dried and then placed onto the L agar plates of *M. luteus* and incubated at 30°C until halos appeared.

Detection of planosporicin by MS. Electrospray ionization (ESI) mass spectrometry (MS) was used to analyze planosporicin production both by full-scan MS analysis for mass determination and by MS-MS fragmentation for sequence analysis (see the supplemental material). Culture supernatants or eluent from Diaion HP20 polystyrene resin were analyzed by matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS (see the supplemental material).

RNA preparation from *P. alba* and RT-PCR. *P. alba* mycelial samples cultured in AF/MS were harvested after 162 h, frozen in liquid N_2 , and ground into a fine powder using a pestle and mortar, and the RNA was extracted with a Qiagen RNeasy minicolumn (see the supplemental material). Two sequential DNase I digestions were performed, yielding 114 μ g RNA; the absence of genomic DNA contamination was confirmed by PCR. RNA (0.73 μ g) was used as the template to synthesize cDNA using Invitrogen Superscript III 1st-strand synthesis Supermix (see the supplemental material). The resulting cDNA was used as a template in a PCR with primers designed to amplify across intergenic regions within the *psp* gene cluster (see the supplemental material and specifically Table S2). The reverse transcription-PCRs (RT-PCRs) used an annealing temperature of 56°C, extension time of 1 min, and 35 cycles. The positive control for each cDNA template used primers annealing within a *P. alba* gene with 87% nucleotide sequence identity (across 1,036 nucleotides) to *S. coelicolor* *hrdB*. *hrdB* encodes the vegetative sigma factor in *Streptomyces* and is commonly used as a control in RT-PCR and quantitative RT-PCR (qRT-PCR) experiments in *Streptomyces* (36).

Manipulation of *P. alba*. *P. alba* exconjugants were generated through conjugations between *E. coli* ET12567/pUZ8002 carrying the ap-

propriate *oriT*-containing cosmid and *P. alba* spores (see the supplemental material). Double-crossover events in deletion mutants were identified through hygromycin resistance and kanamycin sensitivity and were verified by PCR on gDNA from each exconjugant. Mutant phenotypes were complemented by expressing a wild-type copy of the relevant *psp* gene in *trans* either from its own or from the *ermE** promoter (see the supplemental material).

Nucleotide sequence accession number. The sequences reported in this publication for the *psp* gene cluster are available in the EMBL database under accession number [HF570921](#).

RESULTS

Identification of *P. alba* as a planosporicin producer. Four *Planomonospora* strains, *P. alba* NRRL 18924 (25), *P. sphaerica* NRRL 18923 (25), *P. parontospora* subsp. *parontospora* NRRL B-8120 (37), and *P. venezuelensis* NRRL B-16603 (38), were obtained from the ARS culture collection and screened for activity against *Micrococcus luteus*. Only two species, *P. alba* and *P. sphaerica*, produced zones of inhibition on agar plates. In liquid culture, only the supernatant obtained from *P. alba* retained antibacterial activity after passage through a 0.2- μ m Millipore filter, indicating the production of a small bioactive molecule. Production of the antibacterial compound occurred upon entry into stationary phase after approximately 100 h of incubation. ESI mass spectrometry of culture supernatants revealed that *P. alba* produced a compound with the same mass as planosporicin. The monoisotopic mass of the doubly charged $[M + 2H]^{2+}$ ion present in the culture supernatant was 1,096.9027 Da (Fig. 2), equivalent to a mass of 2,191.8054 Da, which corresponds extremely well to the predicted mass of planosporicin of 2,191.7790 Da. Confirmation that the identified compound was indeed planosporicin was obtained by MS-MS fragmentation (Table 2).

Identification of the putative planosporicin biosynthetic gene cluster. Genomic DNA (gDNA) isolated from *P. alba* was sequenced using 454 technology, yielding 73 Mb of assembled

TABLE 2 Major fragments obtained from MS/MS of the 2,191.8054-Da peptide^a

<i>m/z</i>	Residues of core peptide in fragment
1,828.09	5–24(SH)
1,387.08	9–24(SH)
2,076.15	1–23[-Ala(OH)(SH)]
1,470.11	8–24
1,029.05	13–24
1,130.07	12–24
2,079.08	2–24
1,996.13	3–24

^a The fragments are listed as [M + H]⁺ ions in descending order of intensity.

sequence data present in 944 contigs greater than 500 nt in length. The planosporicin core peptide sequence was predicted using the published planosporicin structure (3); dehydroalanine (Dha) and dehydrobutyrine (Dhb) residues were presumed to be derived by dehydration of serine (S) and threonine (T) residues, respectively, and in each (Me)Lan bridge, the cysteine (C) residue was assumed to be carboxy terminal to the dehydrated S or T residues. A search of the *P. alba* 454 contig database using the predicted planosporicin precursor peptide sequence (ITSVSWCTPGCTSEGGGSGCS HSS) and TblastN identified a contig encoding a 56-residue peptide with a 32-residue C-free leader peptide followed by the predicted 24-residue core peptide. The presence of an FQLD motif between positions –17 and –14 (with respect to residue 1 of the core peptide) (Fig. 3) and a conserved proline (P) at position –2 indicated that planosporicin was likely to belong to the class I lantibiotic subfamily (40), implying that two separate enzymes, rather than a bifunctional protein, would carry out the dehydration of S and T residues and subsequent cyclization. Alignment of the planosporicin precursor peptide (PspA) with those of several characterized class I lantibiotics (nisin [41], epidermin [9], microbisporicin [17], and subtilin [42]) as well as several currently uncharacterized putative class I lantibiotics (actoracin [ZP_06162152 from *Actinomyces* oral taxon 848 strain F0332], megateracin [YP_003565954 from *Bacillus megaterium* QM B1551], and nocasporicin [from *Nocardia brasiliensis*]) (Fig. 3) revealed that planosporicin, microbisporicin, actoracin, and nocasporicin form a subclass of more closely related nisin-like lanthipeptides.

Downstream of *pspA* (genes encoding lanthipeptide precursor peptides are generically referred to as *lanA* genes) was a gene encoding the N-terminal region of a homologue of lantibiotic dehydratases (generically referred to as LanB's). A separate TblastN

search of the *P. alba* 454 contig database using SpaB (the dehydratase of the subtilin gene cluster) as the query sequence confirmed the presence of a *lanB* homologue downstream of *pspA* and identified an additional 559-bp contig encoding the central section of a LanB. PCR was used to confirm the proximity of the two contigs in the *P. alba* genome and to provide the 112 bp of intervening nucleotide sequence. The resulting 1,950 bp of contiguous sequence was used to design three hybridization probes covering parts of the putative *lanA*, *lanB*, and *lanAB* genes.

A cosmid library of 3,072 clones was generated from *P. alba* gDNA using the SuperCosI vector (Stratagene). All three probes hybridized to eight cosmid clones that were analyzed by PCR, restriction enzyme digestion, and end sequencing. Two cosmid clones, pIJ12321 and pIJ12322, that appeared likely to contain the entire planosporicin biosynthetic gene cluster were sequenced. The sequence obtained from pIJ12321 was annotated using Artemis (33), revealing a centrally located putative precursor peptide gene, *pspA*, within 37,760 bp of cloned *P. alba* gDNA. pIJ12322 contained 37,916 bp of cloned DNA, with *pspA* located 7.6 kb from one of the junctions with the vector sequence. Amino acid sequences derived from the putative protein coding sequences (PCSs) identified in the cloned DNA using FramePlot (33, 34) were compared with the NCBI protein database using BLASTP (32). Proteins with a significant percent identity to those encoded by the planosporicin gene cluster were used to assign a putative function to each PCS (Table 3).

Heterologous production of planosporicin and identification of a putative minimal gene cluster. To confirm that the two identified cosmid clones contained all of the genes specifically required for planosporicin biosynthesis, attempts were made to express the gene cluster heterologously. pIJ12321 and pIJ12322 were targeted by PCR to introduce an integrase (*intΦC31*), phage attachment site (*attP*), origin of transfer (*oriT*) and apramycin resistance cassette [*aac(3)IV*] into the vector backbone, creating pIJ12323 and pIJ12324, respectively. Each modified cosmid was transferred into *Streptomyces lividans* TK24 and *Streptomyces coelicolor* M1146 by conjugation. Although integration of each cosmid at the cognate attachment site was confirmed by PCR, production of planosporicin was not detected in either *Streptomyces* species using both bioassays against *M. luteus* and MALDI-TOF MS (data not shown). *Nonomuraea* strain ATCC 39727, the producer of the glycopeptide antibiotic A40926 (43), is from the same actinomycete family as *Planomonospora* and had been used previously to express heterologously a lantibiotic gene cluster from another genus of the *Streptosporangiaceae* (17). Thus, both mod-

FIG 3 Alignment (constructed using ClustalW2 [39]) of the planosporicin precursor peptide (PspA) with those of nocasporicin (NspA), microbisporicin (MibA), actoracin (ActA), megateracin (MegA), epidermin (EpiA), nisin (NisA), and subtilin (SpaA). The partially conserved FNLD motif is in bold. The arrow indicates the putative cleavage site of the precursor peptide. Colons indicate amino acids conserved in all eight precursor peptides.

TABLE 3 Putative proteins encoded by the planosporicin biosynthetic gene cluster^a

PCS	<i>psp</i> nomenclature	Length (aa)	Best match (other than <i>mib</i> gene cluster)			Best match in <i>mib</i> gene cluster			Proposed function in <i>psp</i> cluster
			Organism	Accession no.	Annotated function	% Identity (amino acid span)	Accession no.	Annotated function	% Identity (amino acid span)
−9	<i>pspE</i>	269	<i>N. brasiliensis</i>	YP_006806059.1	Hypothetical protein	57 (255)	ADK32561.1	MibE; ABC transporter permease	57 (249)
−8	<i>pspF</i>	249	<i>N. brasiliensis</i>	YP_006806060.1	ABC transporter-like protein	70 (234)	ADK32562.1	MibF; ABC transporter ATP-binding protein	64 (234)
−7	<i>pspR</i>	260	<i>N. brasiliensis</i>	YP_006806057.1	Hypothetical protein	40 (231)	ADK32551.1	MibR; DNA-binding protein	33 (205)
−6	<i>pspQ</i>	138	<i>Thermobispora bispora</i> DSM 43833	YP_003654016.1	Hypothetical protein	56 (129)	ADK32550.1	MibQ; putative lipoprotein	62 (103)
−5	<i>pspZ</i>	301	<i>Eggerthella lenta</i> DSM 2243	YP_003181816.1	ABC transporter-like protein	60 (293)	ADK32548.1	MibZ; ABC transporter ATP-binding protein	59 (288)
−4	<i>pspY</i>	264	<i>N. brasiliensis</i>	YP_006806070.1	Hypothetical protein	38 (247)	ADK32547.1	MibY; ABC transporter permease	39 (213)
−3	<i>pspI</i>	213	<i>Thermobispora bispora</i> DSM 43833	YP_003654019.1	Hypothetical protein	36 (172)	ADK32546.1	MibJ; hypothetical protein	36 (181)
−2	<i>pspW</i>	314	<i>Thermobispora bispora</i> DSM 43833	YP_003654015.1	Hypothetical protein	43 (201)	ADK32552.1	MibW; putative anti-sigma factor	31 (210)
−1	<i>pspX</i>	185	<i>N. brasiliensis</i>	YP_006806067	ECF sigma factor	62 (173)	ADK32553.1	MibX; ECF sigma factor	59 (184)
1	<i>pspA</i>	56	<i>Planomonospora</i> sp. DSM 14920	ACW83043	Structural protein	100 (56)	ADK32554.1	MibA; precursor peptide	31/46 (67%)
2	<i>pspB</i>	1,067	<i>N. brasiliensis</i>	YP_006806066.1	Lantibiotic biosynthesis protein	50 (1063)	ADK32555	MibB; lantibiotic dehydratase	52 (1046)
3	<i>pspC</i>	467	<i>N. brasiliensis</i>	YP_006806065.1	Lanthionine synthetase C-like protein	53 (462)	ADK32556.1	MibC; lantibiotic cyclase	53 (450)
4	<i>pspT</i>	322	<i>N. brasiliensis</i>	YP_006806064.1	ABC transporter ATPase subunit	61 (302)	ADK32558.1	MibT; ABC transporter ATP-binding domain	63 (297)
5	<i>pspU</i>	265	<i>N. brasiliensis</i>	YP_006806063.1	ABC transporter	54 (248)	ADK32559.1	MibU; ABC transporter permease	61 (231)
6	<i>pspV</i>	438	<i>N. brasiliensis</i>	YP_006806062.1	Hypothetical protein	44 (288)	ADK32560.1	MibV; hypothetical protein	42 (324)

^a Each amino acid sequence was used as a query in a NCBI BLASTP search (32) against the nonredundant protein database (as of November 2012). The best match to the proteins encoded by the *mib* gene cluster and the best match to any other protein are listed. aa, amino acids.

M1294 - pIJ10702 (vector)
 M1295 - pIJ12323 (PCS -19 to +14)
 M1299 - pIJ12327 (PCS -19 to *pspV*)
 M1300 - pIJ12328 (PCS -11 to *pspV*)
 M1301 - pIJ12329 (*pspE* to *pspV*)

FIG 4 Heterologous expression of the planosporicin gene cluster integrated into the *Nonomuraea* strain ATCC 39727 chromosome. *Nonomuraea* strains M1294, M1295, M1299, M1300, and M1301 (Table 1) were cultured in SM medium. Supernatant samples were taken after 8 days of growth and tested for antibiotic activity. Forty microliters of supernatant was applied to antibiotic assay discs which were laid upon a lawn of *M. luteus*. The plate was incubated for 36 h at 30°C before zones of inhibition were photographed. The zone of inhibition around strain M1294 is presumed to reflect the production of the glycopeptide antibiotic A40926 by *Nonomuraea* strain ATCC 39727 (43). PCS numbers are relative to *pspA*, where *pspA* is PCS +1.

ified cosmid clones were transferred to *Nonomuraea* strain ATCC 39727 by conjugation. Planosporicin production was readily detected in *Nonomuraea* strain M1295 (containing pIJ12323; two exconjugants tested) when analyzed for activity against *M. luteus*

(Fig. 4) and by MALDI-TOF mass spectrometry (see Fig. S1 in the supplemental material). However, *Nonomuraea* strain M1296 (containing pIJ12324; one exconjugant tested) did not produce the lantibiotic (data not shown). Comparison of the two cloned sequences revealed that pIJ12324 lacked 233 bp of the 3' end of a 1,317-bp PCS that lay at the end of a putative six-gene operon that commenced with *pspA*. It was thus conceivable that this gene, subsequently called *pspV*, was essential for planosporicin production.

To determine the likely boundaries of the planosporicin biosynthetic gene cluster, potentially nonessential genes were removed from the pIJ12321 gDNA insert. PCSs 3' of *pspV* (PCSs +7 to +14, where *pspA* is PCS +1) were predicted to have a role in benzoate metabolism and were deleted from pIJ12321 by PCR targeting. The integrative version of this construct (pIJ12327, containing PCS -19 to *pspV*) still conferred planosporicin production after transfer to *Nonomuraea* (strain M1299) (Fig. 4; see also Fig. S1 in the supplemental material). PCSs at the other end of the gDNA insert of pIJ12321 (PCSs -12 to -19) were predicted to form part of a putative terpenoid biosynthetic cluster. These genes were deleted from pIJ12327 by PCR targeting, and the resulting construct (pIJ12328, containing PCS -11 to *pspV*) was integrated into the *Nonomuraea* chromosome to generate strain M1300, which also produced planosporicin (Fig. 4; see also Fig. S1). A larger region of pIJ12327 was then deleted to remove PCSs -19 to -10, yielding pIJ12329 (containing *pspE* to *pspV*), which, when integrated into the *Nonomuraea* chromosome (strain M1301), also conferred planosporicin production (Fig. 4; see also Fig. S1). Thus, the planosporicin biosynthetic gene cluster appeared to consist of 15 genes contained within a 15.3-kb region of genomic DNA (Fig. 5), a prediction that was subsequently confirmed by deletion analyses (see "Mutational analysis of the planosporicin biosynthetic gene cluster" below). With a GC content of 74.26

FIG 5 Planosporicin gene cluster of *P. alba*. Translationally coupled genes are shaded, and occurrences of translational coupling are indicated by black circles. RT-PCR was used to identify transcriptional units. cDNA (c) was synthesized from RNA extracted from *P. alba* cultured in liquid AF/MS for 162 h and producing planosporicin. Oligonucleotide primers were used to amplify across eight intergenic regions. *P. alba* genomic DNA (g) was used as a positive control for each primer pair. Primers annealing within the presumptive *hrdB* homologue of *P. alba* were used in the absence of reverse transcriptase as a control for genomic DNA contamination. Deduced transcriptional units are indicated by gray arrows.

FIG 6 Syntenous arrangement of homologues of *pspX*, *pspW*, *pspJ*, *pspY*, and *pspZ* in actinomycete genomes. Genes are labeled according to the respective genes from the *psp* cluster, with the locus tag given below. Genes are color-coded by the predicted function of their products: precursor peptides (yellow), biosynthetic enzymes (purple), transporters (dark blue), regulators (light blue), unknown but specific to planosporicin-like gene clusters (green), and unknown (gray).

mol%, the base composition of the cluster corresponds closely to that of the genome of *P. alba* as a whole (73.9 mol% GC).

Bioinformatic analysis of the planosporicin biosynthetic gene cluster. Bioinformatic analysis of the *psp* cluster to assign putative functions to each of the genes was carried out using BLASTP (Table 3). The cluster has notable similarity and synteny both to the *mib* gene cluster of *M. corallina*, encoding the related lantibiotic microbisporicin, and to the as-yet-uncharacterized *nsp* gene cluster from *N. brasiliensis* (Fig. 6). The *N. brasiliensis* gene cluster includes a putative precursor peptide gene, *nspA*, predicted to encode a 24-residue lantibiotic, nocasporicin, with 83% identity to planosporicin.

PspB and PspC are homologous to enzymes that install (Me)Lan bridges. *N. brasiliensis* encodes the proteins with the most similarity to PspB (NspB; 50% identity) and PspC (NspC; 43% identity). The closest homologues from a known lantibiotic gene cluster are MibB (52% identity) and MibC (43% identity), the microbisporicin dehydratase and cyclase from *M. corallina*, respectively. PspB and PspC have lower levels of identity to lantibiotic biosynthetic enzymes from low-GC Gram-positive bacteria (e.g., subtilin's SpaB [23%] and SpaC [29%], epidermin's EpiB [23%] and EpiC [25%], mutacin III's MutB [22%] and MutC [21%], and nisin's NisB [22%] and NisC [26%]). This is consistent with the generally low level (~25% identity) of similar-

ity between members of the LanB family (40). Thus, PspB is predicted to be responsible for the dehydration of the S and T residues present in the planosporicin precursor peptide. LanC enzymes are zinc metalloproteins in which the bound metal ion activates the thiol moiety of the cysteine residue for nucleophilic addition onto dehydrated S (Dha) and T (Dhb) residues. Consistent with this, PspC contains the conserved H and C residues that coordinate the zinc ion in NisC (44). Thus, PspC is proposed to be the cyclase responsible for (Me)Lan bridge formation in planosporicin.

The *psp* gene cluster encodes three putative ABC transporters: PspTU, PspYZ, and PspEF. The ATP-binding and transmembrane (TM) domains of each transporter are present on separate proteins whose genes are cotranscribed (see below and Fig. 5). PspT, PspZ, and PspF are members of the P-loop NTPase superfamily and contain the ATP-binding pocket. The Q-loop of some ATP-binding proteins contains a glutamine residue in a flexible loop that is thought to couple ATP hydrolysis to conformational changes in the TM domain that occur during substrate translocation (45). A subclass of ATP-binding proteins has instead an E-loop, where a glutamic acid replaces the glutamine. In at least some lantibiotic transporters, the E-loop is necessary for immunity toward the produced compound. In NukF of *Staphylococcus warneri* ISK-1, E85Q and E85A substitutions in the E loop impaired immunity to nukacin ISK-1, while ATPase activity remained at wild-type (WT) levels (46). Both PspT and PspZ contain a Q-loop, while PspF contains an E-loop.

PspU, PspY, and PspE are homologues of ABC transporter permeases, and each is predicted to contain six TM helices (TMHMM Server, version 2.0 [47]). Most permease subunits of ABC transporters contain 12 TM domains, so it is conceivable that PspTU, PspYZ, and PspEF function as tetramers of two dimers to form functional ABC transporters. As in other presumptive actinomycete lantibiotic transporters, no putative peptidase domain was found in PspU, PspY, or PspE.

The closest homologues of *pspYZ* are all located next or in close proximity to homologues of *pspXWJ* (Fig. 6), and in at least some cases, they appear to occur in gene clusters that encode small peptides (e.g., in *N. brasiliensis*, *M. corallina*, and *Actinomyces* oral taxon 848 strain F0332). Although the functions of *pspYZ* are not known, their close proximity to *pspXW*, which encode an extracytoplasmic function (ECF) σ -factor and anti-sigma factor, respectively (see below), could imply a role in regulation.

PspR contains a helix-turn-helix domain at its C terminus that is found in members of the LuxR family of transcriptional regulators and may thus be involved in regulation of the *psp* gene cluster. The closest homologues of PspR are O3I_005600 from *N. brasiliensis* and MibR, an essential regulatory protein for microbisporicin production in *M. corallina*.

A second putative regulatory gene is *pspX*, which encodes an ECF σ -factor and contains the rare codon TTA, suggesting that production of planosporicin may be controlled by the *bldA* tRNA and therefore be developmentally regulated (48). The closest homologue of PspX is MibX, which is an activator of microbisporicin biosynthesis in *M. corallina* (49). ECF σ -factors are frequently negatively regulated by membrane-associated anti-sigma factors that are encoded by adjacent and translationally coupled genes (50). Downstream of *pspX*, and translationally coupled to it, is *pspW*. TMpred (51) predicts that PspW contains six transmembrane helices (residues 106 to 292) with a cytoplasmic N terminus (105 amino acids) that could sequester PspX at the membrane.

The closest homologue of PspW is MibW, which has been shown to interact with MibX to negatively regulate microbisporicin production (49). Thus, PspW is predicted to be a negative regulator of planosporicin production. The six closest homologues of PspX are found in *Actinobacteria*, and three of these appear to be in gene clusters that encode lantibiotic biosynthetic enzymes (in *M. corallina*, *N. brasiliensis*, and *Actinomyces* [Fig. 6]).

pspJ encodes a protein of unknown function with several predicted TM helices (TMHMM Server, version 2.0 [47]). All *pspJ* homologues in the current NCBI database lie adjacent to *pspY* homologues and in close proximity to homologues of *pspXWZ* (Fig. 6). PspQ contains a predicted signal peptide sequence containing the lipobox motif LTAC found in lipoproteins (52). The biosynthetic gene clusters for nisin and subtilin also encode lipoproteins (NisI and SpaI, respectively) that provide immunity by sequestering the corresponding lantibiotic, preventing its interaction with lipid II (53, 54). Thus, PspQ could function in concert with PspEF as a self-resistance mechanism against planosporicin. Interestingly, the lipoprotein CseA negatively regulates the ECF sigma factor σ^E in *Streptomyces coelicolor* (55). The proximity of *pspQ* to *pspXW* in *P. alba*, and similar gene arrangements in other known or putative lantibiotic biosynthetic gene clusters (in *N. brasiliensis*, *M. corallina*, and *Thermobispora bispora*), may indicate a role for PspQ in regulating PspX.

pspV encodes a hypothetical protein and has homologues in other known or putative actinomycete lantibiotic biosynthetic gene clusters, e.g., in *M. corallina*, *N. brasiliensis*, *Saccharopolyspora spinosa* NRRL 18395, and *Nocardiopsis dassonvillei* subsp. *dassonvillei* DSM 43111. Other PspV-like proteins occur in streptomycetes, and several are encoded in clusters that also encode a small hypothetical peptide. The closest homologue of PspV is MibV, deletion of which results in loss of chlorination of microbisporicin in *M. corallina* by the halogenase MibH (17). Thus, PspV and its homologues may play roles as scaffolds or chaperones to aid the interaction of lanthipeptide modification enzymes with their cognate precursor peptides.

Transcriptional organization of the planosporicin biosynthetic gene cluster. RNA extracted from *P. alba* mycelium after the onset of planosporicin production was used to establish operon structure within the *psp* gene cluster. A two-step RT-PCR procedure used random hexamers and oligo(dT)₂₀ in an attempt to prime cDNA synthesis in the presence and absence of reverse transcriptase. The resulting cDNA templates were subsequently used with specific primers flanking all 14 intergenic regions within the *psp* gene cluster. Any PCR amplification products generated by these oligonucleotides would therefore be indicative of transcription across the intergenic region. The results obtained (Fig. 5 shows most of the analyses) demonstrated the existence of three operons in the *psp* gene cluster: *pspABCTUV*, *pspXWJYZQR*, and *pspEF*. This analysis does not preclude the existence of additional promoters, particularly in the larger intergenic regions between *pspW* and *pspJ* (231 bp) and between *pspQ* and *pspR* (89 bp). Indeed, when the number of PCR cycles was reduced from 35 to 30, the transcript spanning *pspWJ* was no longer detected, while those spanning *pspJYZQR* were, implying that *pspJ* may have an additional promoter. Control experiments showed that the RNA prepared from *P. alba* mycelium was free from contamination with genomic DNA; no amplification of *P. alba hrdB* was observed when cDNA synthesis was attempted in the absence of reverse transcriptase. The fidelity of the oligonucleotide primers was con-

FIG 7 Bioassays of the *P. alba* deletion mutants. Strains M1308 (Δ *pspR::hyg*), M1310 (Δ *pspQ::hyg-oriT*), M1305 (Δ *pspYZ::hyg-oriT*), M1309 (Δ *pspI::hyg-oriT*), M1304 (Δ *pspW::hyg*), M1303 (Δ *pspX::hyg*), M1302 (Δ *pspA::hyg-oriT*), M1306 (Δ *pspTU::hyg-oriT*), and M1307 (Δ *pspV::hyg-oriT*) were grown in AF/MS liquid medium for 6 days alongside WT *P. alba* as a positive control. Samples of culture supernatants were assayed for antibiotic activity by spotting 40 μ l of supernatant onto antibiotic assay discs which were laid onto a lawn of *M. luteus*. The plate was incubated for 36 h at 30°C before zones of inhibition were photographed. Planosporicin production was also assessed by MALDI-TOF mass spectrometry (see Fig. S2 in the supplemental material).

firmed through the presence of amplicons of the same size when using genomic DNA as the template.

Mutational analysis of the planosporicin biosynthetic gene cluster. Gene deletions in actinomycetes are generally accomplished by homologous recombination after introducing nonreplicative and appropriately engineered cosmid or plasmid constructs from *E. coli* by conjugation. Preliminary experiments revealed that both the Φ BT1 *attB* and Φ C31 *attB* sites were present in the *P. alba* genome for plasmid integration and subsequent mutant complementation, and that both apramycin and hygromycin could be used effectively to select for exconjugants (data not shown). A conjugation protocol was thus developed (see the supplemental material) and used to create a series of gene replacements in the planosporicin biosynthetic gene cluster.

To confirm that the identified gene cluster did indeed encode planosporicin biosynthesis, pIJ12340, in which *pspA* encoding the putative planosporicin precursor peptide had been replaced by a hygromycin resistance cassette, was conjugated into *P. alba* to create three hygromycin-resistant, kanamycin-sensitive double-crossover recombinants (kanamycin resistance was encoded by the delivery plasmid). Deletion of *pspA* was confirmed in each case by PCR and resulted in loss of planosporicin biosynthesis when the mutants (e.g., M1302) were analyzed by bioassay against *M. luteus* (Fig. 7) and by MALDI-TOF MS (see Fig. S2 in the supplemental material), confirming the identity of the *psp* gene cluster.

A similar approach was used to create eight further mutants that targeted putative planosporicin biosynthetic genes. Unless otherwise stated, gene replacement was achieved by homologous recombination using the PCR-targeted minimal gene set present in pIJ12328. At least three independent mutant clones (unless otherwise stated) were verified by PCR and then assessed for planosporicin production using bioassays with *M. luteus* (with both agar- and liquid-grown cultures of *P. alba*) and MALDI-TOF MS.

Bioinformatic analysis had revealed three putative regulatory genes in the *psp* cluster, *pspR*, *pspX*, and *pspW*. Deletion of *pspR* abolished planosporicin biosynthesis (two clones tested; one, M1308, is shown in Fig. 7 and in Fig. S2 in the supplemental material), which was restored by expressing *pspR* in *trans* from the *ermE** promoter after integration at the Φ C31 *attB* site (see Fig. S3 in the supplemental material). Thus, PspR, a putative DNA-binding protein, appears to be an activator of lantibiotic biosynthesis. Deletion of *pspX* also abolished planosporicin biosynthesis (e.g., M1303 in Fig. 7 and in Fig. S2 in the supplemental material), which was subsequently restored in the mutant by expressing *pspX* in *trans* from its own promoter after integration at the Φ C31 *attB* site (see Fig. S3), similarly confirming that the ECF σ factor is a positively acting regulatory protein. In contrast to the previous two mutations, deletion of *pspW* appeared to markedly increase the level of planosporicin production (e.g., M1304 in Fig. 7 and in Fig. S2), consistent with its putative role as a negative regulator of *pspX*. This overproduction phenotype was partially complemented in *trans* by expressing *pspW* from its native promoter after integration at the Φ C31 *attB* site (see Fig. S3).

Deletion of *pspV* was achieved through homologous recombination using a plasmid containing Δ *pspV::hyg-oriT* flanked by 2 kb of sequence homologous to the *psp* cluster (two exconjugants tested). Deletion of this gene of unknown function abolished planosporicin production (e.g., M1307 in Fig. 7 and in Fig. S2 in the supplemental material), which was restored by expressing *pspV* in *trans* from the *ermE** promoter after integration at the Φ C31 *attB* site (see Fig. S3). This is consistent with the phenotype observed in *Nonomuraea* strain M1296, which lacked a complete copy of the gene. Deletion of *pspTU* (achieved by homologous recombination using cosmid pIJ12321; one exconjugant tested), which encode a putative ABC transporter, also abolished antibiotic activity (M1306 in Fig. 7), and MALDI-TOF analysis of both culture supernatants (see Fig. S2) and mycelial extracts (data not shown) failed to reveal the presence of the lantibiotic, implying that planosporicin was not accumulating in the mycelium. Given that *pspV* is also required for planosporicin biosynthesis, it is conceivable that the phenotype of the *pspTU* mutant reflects a polar effect on *pspV* expression. However, the hygromycin resistance cassette used to replace *pspTU* contains the promoter of the apramycin resistance gene reading into *pspV*, thus potentially overriding polarity. Thus, the nonproducing phenotype may indeed reflect deletion of the ABC transporter.

Deletion of *pspQ*, encoding a putative lipopeptide, had no effect on planosporicin production (e.g., M1310 in Fig. 7 and in Fig. S2 in the supplemental material). In contrast, deletion of *pspYZ*, also encoding a putative ABC transporter, severely reduced lantibiotic production. Planosporicin was not detected in most assays of supernatants from liquid-grown cultures, either in bioassays (e.g., M1305 in Fig. 7) or by MALDI-TOF analysis (see Fig. S2). Occasionally a small zone of inhibition was observed and planosporicin production confirmed by MALDI-TOF mass spectrometry (data not shown). Bioassays of agar-grown cultures were more reproducible, exhibiting a small zone of inhibition at earlier time points than with the wild-type strain which did not increase over time (data not shown). Deletion of *pspJ* also abolished planosporicin production (e.g., M1309 in Fig. 7 and in Fig. S2). Despite repeated attempts, it was not possible to obtain a *pspEF* replacement mutant using the same strategy, potentially indicating an essential role in conferring immunity in the producing organism.

FIG 8 Expression of *pspEF* in *S. coelicolor* M1152 confers immunity to planosporicin. The *P. alba* WT and strains M1302 (Δ *pspA*) and M1304 (Δ *pspW*) were streaked in duplicate on AF/MS agar. After 4 days, either (i) the strains were overlaid with *M. luteus* or (ii) streaks of *S. coelicolor* M1152 and M1553 (*ermE***p-pspEF*) were added perpendicular to the *P. alba* streaks. Plates were photographed after a further 4 days of incubation.

Expression of *pspEF* in *S. coelicolor* confers resistance to planosporicin. The closest homologues of PspEF are MibEF from *M. corallina*, which appear to confer immunity to microbisporicin (17). To assess whether *pspEF* might function to confer immunity to planosporicin, the genes were cloned downstream of the strong constitutive *ermE** promoter in pIJ10257, creating pIJ12717, which was then integrated at the Φ BT1 site of *S. coelicolor* M1152 to yield M1553 (Table 1). Expression of *pspEF* in *S. coelicolor* M1152 clearly conferred some level of immunity to planosporicin (Fig. 8). The same phenotype was observed with concentrated *P. alba* supernatant (data not shown). Thus, PspEF may confer immunity to planosporicin in *P. alba* by diminishing the concentration of the lantibiotic in proximity to its likely target, lipid II, in the cell envelope (56).

DISCUSSION

P. alba produces the pentacyclic lantibiotic planosporicin, in which 50% of the 24 amino acids are subject to posttranslational modification. Revision of the planosporicin structure from that published in reference 22 to that in reference 3 involved a shift of two amino acids and a reorganization of two of the Lan bridges.

This revised structure was confirmed in this study by sequencing the gene encoding the planosporicin precursor peptide and by MS-MS analysis of the mature lantibiotic.

The bioinformatic and deletion analyses reported here provide substantial insights into the biosynthesis of planosporicin. Nevertheless, significant questions remain. For example, why does the cluster encode three putative ABC transporters? Replacement of *pspTU* with the hygromycin resistance cassette resulted in loss of planosporicin biosynthesis. While this could reflect a polar effect on the expression of *pspV*, which is located downstream of *pspTU* in the *pspABCTUV* operon and essential for planosporicin production, replacement of *pspQ* in the *pspJWZQR* operon with the same cassette in the same relative orientation did not have a polar effect on the expression of *pspR*, which is essential for planosporicin production. This is attributed to the outward-reading apramycin resistance gene promoter, which would transcribe both *pspR* and *pspV* in the corresponding deletion mutants. Thus, *pspTU* may well be essential for planosporicin production, and the location of these genes in the biosynthetic *pspABCTUV* operon would seem consistent with a role in transporting the antibiotic immediately after synthesis into the extracellular environment. Indeed, there is precedence for the deletion of ABC transporter genes preventing lantibiotic production (57). However, there are also examples where deletion of putative transporter genes had no apparent effect on lantibiotic production (42, 58), perhaps most notably in the related microbisporicin biosynthetic gene cluster (17). In such cases, export of the lantibiotic was attributed to other transporters located either in the same gene cluster or elsewhere on the chromosome.

Deletion of the *pspYZ* ABC transporter genes severely reduced planosporicin production, and deletion of *pspJ*, a gene of unknown function, abolished it completely. The more severe phenotype of the latter mutants, and the ability of the *pspQ* replacement mutants to produce planosporicin, implies lack of polarity in the *pspJYZQR* operon mutants, again potentially attributable to the outwardly reading apramycin promoter present in the hygromycin resistance cassette. A very low level of planosporicin production was detected in the *pspYZ* mutants in agar-grown cultures, indicating that the genes are not absolutely required for production. If a major role of PspYZ is to export the lantibiotic, then, given the small quantities found outside the mycelium, it seemed possible that the mutants would accumulate planosporicin (possibly still attached to its leader peptide) intracellularly. Failure to detect planosporicin in the mycelium of either the *pspYZ* or *pspJ* mutants (data not shown) suggests that PspYZ do not play a major role in planosporicin export. The close proximity of *pspJYZ* homologues to *pspXW* homologues in several actinobacteria suggests that they may instead be involved in regulating the activity of PspX.

Despite repeated attempts, it was not possible to delete *pspEF* using the same hygromycin resistance cassette. The heterologous expression of *pspEF* in *S. coelicolor* indicates that this ABC transporter can confer some resistance to planosporicin. In *P. alba*, PspEF may play an essential role in immunity to planosporicin; i.e., deletion would be lethal, yet the homologous *mibEF* genes present in the microbisporicin biosynthetic cluster could be deleted (17), although this resulted in loss of microbisporicin production. This might reflect different regulatory mechanisms in the two strains that coordinate immunity with production. The re-

sults presented in this article pave the way for a further detailed analysis of the regulation of planosporicin biosynthesis in *P. alba*.

ACKNOWLEDGMENTS

We thank Govind Chandra for assembling the 454 database and assistance with bioinformatics; Lucy Foulston, Gorgia Letizia Marcone, and Flavia Marinelli for advice on the manipulation of *Nonomuraea*; Lucy Foulston and Sean O'Rourke for technical advice; and Gerhard Saalbach and Mike Naldrett for mass spectrometry.

This work was supported financially by the Biotechnological and Biological Sciences Research Council (BBSRC) Institute (United Kingdom) Strategic Programme Grant "Understanding and Exploiting Plant and Microbial Secondary Metabolism" (BB/J004561/1) and the John Innes Foundation. E.J.S. was supported by a BBSRC doctoral training grant.

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