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Distinct mechanisms contribute to immunity in the lantibiotic NAI-107 producer strain *Microbispora* ATCC PTA-5024

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Abstract

The investigation of self-resistance in antibiotic producers is important to understand the emergence of antibiotic resistance in pathogens and to improve antibiotic production. Lantibiotics are ribosomally synthesized antibiotics that mostly target peptidoglycan biosynthesis. The actinomycete *Microbispora* ATCC PTA-5024 produces the lantibiotic NAI-107, which interferes with peptidoglycan biosynthesis by binding bactoprenol-pyrophosphate-coupled peptidoglycan precursors. In order to understand how *Microbispora* counteracts the action of its own antibiotic, its peptidoglycan composition was analysed in detail. *Microbispora* peptidoglycan consists of muropeptides with D-Ala and Gly in similar proportion at the fourth position of the peptide stems and alternative 3-3 cross-links besides the classical 4-3 cross-links. In addition, the NAI-107 biosynthetic gene cluster (*mlb*) was analysed for the expression of immunity proteins. We show that distinct immunity determinants are encoded in the *mlb* cluster: the ABC transporter MlbYZ acting cooperatively with the transmembrane protein MlbJ and the lipoprotein MlbQ. NMR structural analysis of MlbQ revealed a hydrophobic surface patch, which is proposed to bind the cognate lantibiotic. This study demonstrates that immunity in *Microbispora* is not only based on one determinant but on the action of the distinct immunity proteins MlbQ, MlbYZ and MlbJ.

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Introduction

The development of antibiotic resistance in clinically relevant pathogens is one major obstacle to effective treatment of bacterial infections and the main reason for the urgent need of new therapeutic strategies. Among the most exploited classes of antibiotics used against Gram-positive bacteria, beta (β)-lactams and glycopeptides have played a predominant role in antibiotic chemotherapy. Their widespread use has led to the emergence of resistant pathogenic bacteria, and several mechanisms conferring resistance have been well documented (Rice, 2012). Both β-lactams and glycopeptides are cell wall-targeting antibiotics which interfere with the peptidoglycan biosynthesis, an essential process in viable cells. Peptidoglycan (PG) is a polymer that surrounds the cytoplasmic membrane preserving the cell integrity from the turgor and maintaining the cell shape. It consists of linear glycan chains of repeated N-acetylglucosamine-N-acetylmuramic acid disaccharide units (GlcNAc-MurNAc) that are interconnected by peptide cross-links. The key component of PG synthesis is lipid II which consists of the disaccharide GlcNAc-MurNAc loaded onto the lipid carrier undecaprenyl pyrophosphate and linked to a pentapeptide, typically L-Ala-γ-D-Glu-X-D-Ala-D-Ala, where X is *meso*-A_2pm (2,6-diaminopimelic acid) or L-Lys. After the synthesis in the cytoplasm, lipid II is translocated and exposed to the outer leaflet of the cytoplasmic membrane where it is incorporated in the nascent glycan chains by transglycosylases and processed by transpeptidases which cross-link the peptide side-chains of adjacent glycan strands (Vollmer *et al.*, 2008). Diversification of the PG assembly pathway leading to β-lactam and glycopeptide resistance had been widely described. The expression of penicillin-binding proteins (PBPs) with low affinity to β-lactams and the synthesis of glycopeptide-resistant PG precursors with pentapeptides

ending in D-Lac or D-Ser instead of D-Ala are two of the major resistance mechanisms found in Gram-positive bacteria (Rice, 2012). Moreover, L,D-transpeptidases confer resistance both to β-lactams and glycopeptides by bypassing the β-lactam sensitive D,D-transpeptidase activity of PBPs and using tetrapeptide acyl donors devoid of the target of glycopeptides respectively (Mainardi *et al*., 2005; 2008). Despite the emergence of resistance to these two classes of antibiotics, PG biosynthesis remains an effective target for the development of new antibacterial strategies. The PG precursor lipid II, the bottleneck of cell wall synthesis, is the target not only of glycopeptides but also of lantibiotics, mannopeptimycins and ramoplanin (Breukink and de Kruijff, 2006). Lantibiotics are attractive drug candidates due to their potency and broad-spectrum activity. A few lantibiotics are currently in preclinical development or in clinical trials for the treatment of infections caused by Gram-positive bacteria (Jabes *et al*., 2011; Crowther *et al*., 2013; Sandiford, 2015).

Lantibiotics are ribosomally synthesized and post-translationally modified peptides which display antimicrobial activity. According to the new nomenclature, lantibiotics are classified into the lanthipeptide family, which comprises lanthionine-containing peptides regardless of their biological activities (Arnison *et al*., 2013). Lanthipeptides are characterized by the presence of the thioether-cross-linked amino acids lanthionine (Lan) and 3-methyl-lanthionine (MeLan) that are required for activity and confer protease resistance to the mature product. The cross-linked amino acids are formed by dehydration of serine or threonine residues and intramolecular addition of a cysteinyl thiol to the resulting dehydroamino acids (Knerr and van der Donk, 2012). Lanthipeptides are classified into four classes according to the biosynthetic enzymes that introduce the Lan and MeLan motifs. The most extensively studied lantibiotic, nisin, belongs to the class I lanthipeptides, which are modified by the two distinct enzymes LanB (dehydratase) and LanC (cyclase). Class I lanthipeptide biosynthetic gene clusters encode the precursor peptide and the enzymes involved in lanthionines formation, transporters, proteases, modifying enzymes, regulators and immunity proteins (Willey and van der Donk, 2007). Many of the lantibiotics display their antibacterial activity by binding the PG precursor lipid II, and therefore interfering with the PG biosynthesis (Broetz *et al*., 1998; Bierbaum and Sahl, 2009). In addition, nisin can permeabilize bacterial membranes by lipid II-dependent pore formation and cause the dissipation of the membrane potential and rapid efflux of small metabolites (Wiedemann *et al*., 2001).

Lantibiotic producers express immunity proteins like ABC transporters, lipoproteins, membrane-associated peptides and transmembrane proteins as a measure of self-protection (Okuda and Sonomoto, 2011). However, despite the expression of immunity proteins, lantibiotic-producer strains do not exhibit a full resistance, and the susceptibility to their own products may reflect the efficacy of these antibacterials. Indeed, lantibiotic autotoxicity has represented an obstacle for the improvement of fermentation processes (Valsesia *et al*., 2007). In many antibiotic-producing bacteria, antibiotic resistance is mediated by ABC transporters, which export the drugs through the cytoplasmic membrane (Mendez and Salas, 2001). As lantibiotics interact with lipid II located on the cell surface, ABC transporters involved in lantibiotic immunity should have another mechanism. A cell-based transport assay reported for EpiEFG showed that this ABC transporter system can extrude the membrane-bound epidermin to the extracellular space, decreasing the concentration of the lantibiotic in the lipid II immediate environment (Otto *et al*., 1998). In contrast to the epidermin cluster (*epi*), the nisin (*nis*) and subtilin (*spa*) gene clusters encode the lipoproteins NisI and SpaI that confer immunity to the producers in addition to the ABC transporters NisFEG and SpaFEG, homologues of EpiEFG. The lantibiotics nisin and subtilin have similar structures; however, the corresponding immunity lipoproteins NisI and SpaI do not show any sequence similarity and they do not confer cross-immunity. Although the expression of these genes in surrogate hosts clearly showed an involvement in immunity (Stein *et al*., 2003; 2005), their mechanism of action on a molecular level is still largely unknown.

NAI-107 is a lantibiotic produced by the actinomycete *Microbispora* ATCC PTA-5024, active against Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus*, glycopeptide-intermediate *S. aureus*, vancomycin-resistant enterococci and some Gram-negative bacteria (Lazzarini *et al*., 2006; Castiglione *et al*., 2008). Because of its interesting antibacterial profile and potent antibacterial activity, NAI-107 is currently in the preclinical development for the treatment of multi-drug-resistant Gram-positive pathogens (Jabes *et al*., 2011; Lepak *et al*., 2015). NAI-107 inhibits PG biosynthesis and affects the bacterial membrane by slow depolarization (Muench *et al*., 2014). Macromolecular synthesis, whole-cell and enzyme assays combined with proteomic profiling showed that NAI-107 targets lipid II but it does not display a pore-forming activity (Muench *et al*., 2014), as previously described for nisin. NAI-107, also called microbisporicin, was the first discovered class I lantibiotic produced by actinomycetes. It includes one methyl-lanthionine, three lanthionines and two unusual lantibiotic modifications: a chlorinated tryptophan and a mono- or dihydroxylated proline (Lee, 2003; Castiglione *et al*., 2008). The N-terminal lanthionine rings (1-11 amino acids) resemble the lipid II-binding motif of nisin (Hsu *et al*., 2004), whereas the C-terminal aminovinyl cysteine is also found in epidermin (Allgaier *et al*., 1985). In addition to

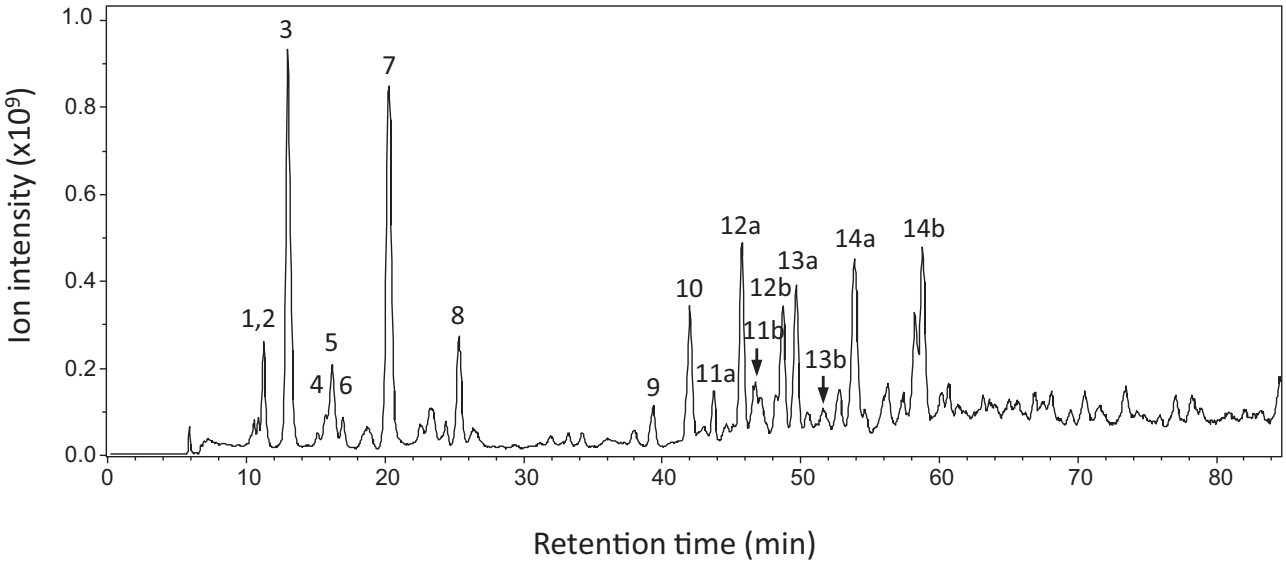

Fig. 1. High-performance liquid chromatography–mass spectrometry chromatogram of the muropeptides from *Microbispora* ATCC PTA-5024 (positive mode). Numbers refer to Table 1. Peaks 1 and 2 overlap. Peaks a and b correspond to muropeptides with the same *m/z*.

Microbispora ATCC PTA-5024, another strain, *Microbispora corallina* NRRL 30420, was described to produce microbisporicin (Lee, 2003; Foulston and Bibb, 2010). Recently, the congeners of the NAI-107 complex produced by *Microbispora* PTA-5024 and *M. corallina* NRRL 30420 were characterized (Maffioli *et al*., 2014). The genes responsible for the biosynthesis of microbisporicin were widely described in the producer *M. corallina* NRRL 30420 (Foulston and Bibb, 2010). The *mib* cluster encodes two enzymes not described before in lantibiotic gene clusters: a cytochrome P450 (MibO) and a flavin-dependent tryptophan halogenase (MibH) involved in hydroxylation of proline and chlorination of tryptophan respectively Furthermore, the cluster encodes three putative ABC transporters (MibYZ, MibTU, MibEF), a predicted lipoprotein (MibQ) and a transmembrane protein of unknown function (MibJ). Recently, the draft genome sequence of *Microbispora* ATCC PTA-5024 was published (Sosio *et al*., 2014). The screening of *Microbispora* ATCC PTA-5024 genome revealed the presence of a gene cluster with high similarity to the *mib* cluster, named *mlb* cluster (Donadio *et al*., 2009).

Although NAI-107 was tested in several *in vitro* studies against multi-drug-resistant pathogens, no resistant mutants were observed. The analysis of NAI-107 resistance in *Microbispora* ATCC PTA-5024 was therefore conducted to determine the mechanisms evolved in the producer strain to protect itself from NAI-107 and predict possible resistance mechanisms in pathogenic bacteria. We used two different approaches to study *Microbispora* immunity: a detailed analysis of the PG, target of NAI-107 and the investigation of resistance determinants encoded in the NAI-107 biosynthetic gene cluster (*mlb*). In particular, we analysed the immunity lipoprotein MlbQ and solved the structure of MlbQ by NMR. This study represents the first comprehensive analysis of immunity in a lantibiotic-producing actinomycete.

Results and discussion

The identification of 3-3-cross-linked muropeptides in Microbispora *ATCC PTA-5024 suggests the action of L,D-transpeptidases in peptidoglycan synthesis*

The lantibiotic NAI-107 interferes with the late stages of PG biosynthesis by binding the bactoprenol-pyrophosphate coupled PG precursors (Muench *et al*., 2014). In order to investigate possible PG modifications responsible for the protection of *Microbispora* ATCC PTA-5024 from NAI-107, a detailed PG structural analysis was performed combining high performance liquid chromatography, mass spectrometry and amino acid analysis. The structure of muropeptide monomers was confirmed by tandem mass spectrometry (MS^2 and MS^3).

Mass spectrometry analyses of mutanolysin digested PG from exponentially growing bacteria (72 h) revealed the presence of muropeptide monomers and dimers with a structural variability (Fig. 1, Table 1). Besides the presence of pentapeptides (8) (pentapeptide = GlcNAc-MurNAc-L-Ala-γ-D-iGln-*meso*-A_2pm-D-Ala-D-Ala), tripeptides (1) and tetrapeptides (7) peptides were detected (Fig. 1, Table 1). The muropeptide monomers from *Microbispora* ATCC PTA-5024 were fully amidated at Glu^2 (Table 1). Furthermore, monomeric tetrapeptides ending in Gly instead of Ala were detected (Fig. 1; Table 1; Fig. S1).

Table 1. Proposed structure of the muropeptides from *Microbispora* ATCC PTA-5024.

		m/z [M+H]$^+$	
Muropeptides[a]	Proposed structure[b]	Observed	Calculated
1	Tri	870.4	870.39
2	Tetra(Ser)	957.5	957.42
3	Tetra (Gly)	927.4	927.42
4	Penta(Ser)	1028.5	1028.46
5	Di	698.3	698.31
6	Penta(Gly)	998.5	998.45
7	Tetra(Ala)	941.4	941.43
8	Penta(Ala)	1012.5	1012.47
9	Tri-Tetra(Ser)[d]	1808.9	1808.80
10	Tri-Tetra(Gly)[d]	1778.9	1778.79
11a,b[c]	Tetra-Tetra(Ser) and Tri-Penta(Ser)[d]	1879.9	1879.84
12a,b[c]	Tetra-Tetra(Gly) and Tri-Penta(Gly)[d]	1849.9	1849.83
13a,b[c]	Tetra-Tri and Tri-Tetra(Ala)[d]	1792.8	1792.81
14a,b[c]	Tetra-Tetra(Ala) and Tri-Penta(Ala)[d]	1863.8	1863.84

a. Muropeptide numbers refer to Fig. 1.
b. In brackets, the amino acids in the fourth or fifth position in monomers and in the acceptor peptide in dimers. The dimers are indicated with the donor and acceptor peptides.
c. Muropeptides a and b have the same *m/z*.
d. Dimers with a proposed 3-3 cross-link.

The presence of Gly in the monomer tetrapeptide 3 at *m/z* 927.4 is presumably the result of a modification that occurs in the late stages of PG maturation, since no UDP-linked PG precursors containing Gly in the fourth position of the peptide were detected in *Microbispora*. Tetrapeptides ending in D-Ser (2) (Fig. 1, Table 1) were also detected in *Microbispora* PG, though in minor amounts. The exchange of D-Ala^4 for Gly or other D-amino acids is known to occur as a side reaction of L,D-transpeptidases, β-lactam-resistant enzymes first described in *Enterococcus faecium*, where they cross-link the PG using tetrapeptides as acyl donor (Mainardi *et al*., 2005). The presence of several genes in the *Microbispora* ATCC PTA-5024 genome (Sosio *et al*., 2014) that encode putative L,D-transpeptidases may explain the ability of *Microbispora* to substitute D-Ala^4 for Gly or D-Ser in monomer tetrapeptides.

The modifications described for the muropeptide monomers could also be detected for the dimers, which contain Ala (13, 14), Gly (10, 12) or Ser (9, 11) in the fourth or fifth position of the acceptor peptides (Fig. 1, Table 1). According to the *m/z* values, muropeptides with 4-3 cross-links resulting from a D,D-transpeptidase activity were suggested (Table 1). Interestingly, the structure of the dimers 9 and 10 could be explained exclusively by the presence of 3-3 cross-links rather than 4-3 cross-links. Dimers with a disaccharide tripeptide as acyl donor were in fact considered to contain direct 3-3 cross-links. Moreover, the presence of two isomers (a and b) for the muropeptides at *m/z* 1879.9, 1849.9, 1792.8 and 1863.8 (11, 12, 13 and 14) may be the result of the two cross-link types (Fig. 1, Table 1). The modifications found in muropeptide monomers and dimers suggest the action of both D,D- and L,D-transpeptidases in *Microbispora* PG cross-linking and remodelling.

The activity of L,D-transpeptidases was previously reported for some members of the order *Actinomycetales* (Lavollay *et al*., 2008; Hugonnet *et al*., 2014) but not described before for lantibiotic producers.

The non-producer strains Microbispora *RP0 and* Microbispora *JCM 10266 exhibit the same muropeptides as the NAI-107 producer* Microbispora *ATCC PTA-5024*

To determine if the PG composition of *Microbispora* ATCC PTA-5024 is linked to NAI-107 production, a non-producer strain was constructed. Since *Microbispora* ATCC PTA-5024 is not genetically accessible, a gene transfer system had to be developed. DNA transfer could be achieved by using a conjugation protocol based on the methylation-proficient donor *Escherichia coli* S17-1, apramycin for the selection of *Microbispora* ex-conjugants and phosphomycin for the inhibition of *E. coli* (see *Experimental procedures*).

The non-producer strain *Microbispora* RP0 was obtained by disruption of the NAI-107 biosynthetic gene cluster (*mlb*) by integration of the plasmid pGusA21-*mlbAB*. The β-glucuronidase (GUS) reporter system allowed an easy identification of recombinant clones carrying the plasmid (see *Experimental procedures*). Using bioassays and high-performance liquid chromatography–mass spectrometry (HPLC-MS) analysis, we proved that the mutant *Microbispora* RP0 does not produce any NAI-107. The PG of *Microbispora* RP0 was isolated at 72 h and was analysed by HPLC. The HPLC profile of *Microbispora* RP0 muropeptides at 72 h was similar to that of the wild type (WT) (Fig. S2). Mass spectrometry analysis revealed the presence of the same PG modifications (data not shown). Thus, the PG composition of *Microbispora* RP0 suggested a PG structure that is independent from NAI-107 production. To determine if the variability of PG muropeptides is characteristic of *Microbispora* ATCC PTA-5024, we analysed the PG of *Microbispora* JCM 10266, a *Microbispora* sp. that produces neither the lantibiotic NAI-107 nor any other antibacterial compound. Analysis of *Microbispora* JCM 10266 PG by mass spectrometry did not show any significant difference to the PG of *Microbispora* ATCC PTA-5024 (data not shown). The PG modifications found in *Microbispora* spp. seemed to be an intrinsic characteristic of *Microbispora* genus, rather than a mechanism of NAI-107 resistance. Peptidoglycan remodelling in

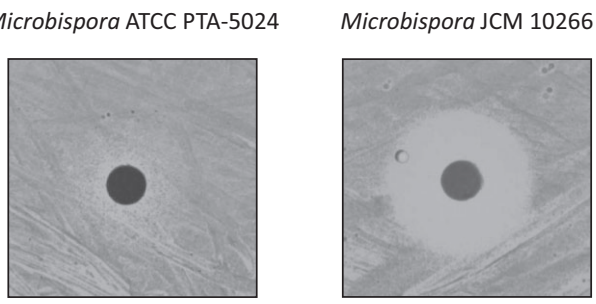

Fig. 2. Resistance of *Microbispora* ATCC PTA-5024, the producer of NAI-107 and the non-producer strain *Microbispora* JCM 10266 to NAI-107 (1 μg).

Microbispora could be a general attempt to counteract the action of cell wall-targeting antibiotics produced by soil-dwelling bacteria.

The mlb *cluster encodes resistance determinants*

The occurrence of the same PG structure in the NAI-107 producer and in non-producing *Microbispora* strains excludes target modification as a specific mechanism of self-protection against NAI-107. To analyse the NAI-107 immunity mechanisms in the producer strain *Microbispora* ATCC PTA-5024, we first compared its resistance level to that of *Microbispora* JCM 10266 by bioassays. *Microbispora* ATCC PTA-5024 was considerably more resistant to NAI-107 than the non-producer strain *Microbispora* JCM 10266 (Fig. 2), which does not contain the *mlb* cluster (*Microbispora* ATCC PTA-5024 MIC 0.5–1 μg ml^{-1}, *Microbispora* JCM 10266 MIC 0.1 μg ml^{-1}). Thus, the difference in resistance between the two *Microbispora* spp. is presumably due to the action of immunity proteins encoded in the NAI-107 gene cluster (*mlb*). Bioinformatic analysis of the *mlb* cluster led to the identification of the putative immunity proteins MlbQ, MlbYZ, MlbTU and MlbEF. MlbQ is a lipoprotein of 129 amino acids with a conserved lipobox (LAGC) containing a cysteine, which presumably becomes the N-terminal residue of the mature protein (amino acids 24–129) after lipidation and cleavage of the signal peptide. MlbYZ, MlbTU, MlbEF are putative ABC transporters which are composed of a permease (MlbY, MlbU, MlbE) and an ATPase (MlbZ, MlbT, MlbF) that probably assemble as tetramers of two heterodimers.

To assess the role of these proteins in NAI-107 resistance, *mlbQ*, *mlbYZ*, *mlbTU* and *mlbEF* were heterologously expressed in *Streptomyces coelicolor* M145. The genes were cloned into the integrative plasmid pRM4 under the control of the constitutive promoter *ermE*p* and resistance assays against NAI-107 were performed. *Streptomyces coelicolor* M145 expressing the lipoprotein MlbQ was more resistant to NAI-107 than the WT (Fig. 3A). To confirm the result, *mlbQ* was expressed in *Microbispora* JCM 10266, a closely related strain of the NAI-107 producer. MlbQ conferred NAI-107 resistance also to this strain (data not shown). In contrast to MlbQ, none of the ABC transporters encoded in the *mlb* cluster conferred NAI-107 protection in *S. coelicolor* (Fig. 3B). This is in contrast to the experiments described for the MlbEF homologues MibEF and PspEF for which a role in immunity was reported. MibEF and PspEF are ABC transporters encoded in the microbisporicin and planosporicin clusters from the actinomycete species *Microbispora corallina* and *Planomonospora alba* respectively (Foulston and Bibb, 2010; Sherwood *et al.*, 2013).

The ABC transporter MlbYZ and the transmembrane protein MlbJ confer immunity to NAI-107 cooperatively

Several ABC transporters of antimicrobial peptides are functional in association with accessory proteins whose mechanism of action is mostly unknown (Gebhard, 2012).

To determine whether any of the ABC transporters in the *mlb* cluster is involved in resistance by acting in cooperation with another protein, we performed coexpression studies. MlbYZ, MlbTU, MlbEF were coexpressed in the heterologous host *S. coelicolor* M145

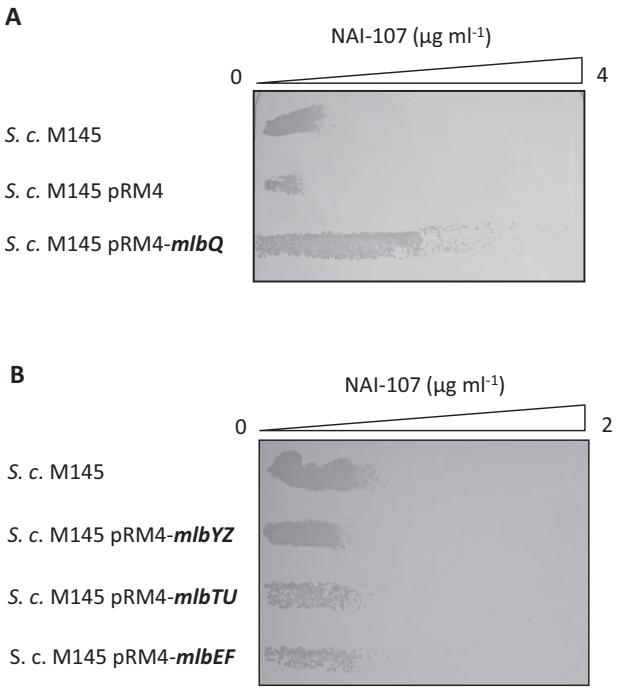

Fig. 3. Resistance of *S. coelicolor* M145 (*S. c.*) recombinant strains expressing the lipoprotein- and the ABC transporter genes to NAI-107.
A. *Streptomyces coelicolor* WT, containing the empty vector pRM4 and expressing *mlbQ*.
B. *Streptomyces coelicolor* WT and expressing *mlbYZ*, *mlbTU* or *mlbEF*. NAI-107 gradient plates (0–4, 0–2 μg ml^{-1}).

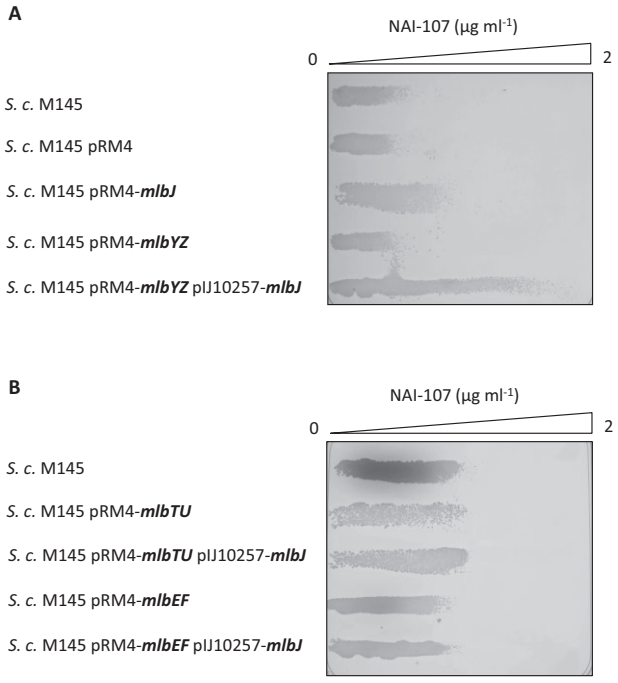

Fig. 4. Resistance of *S. coelicolor* M145 (*S. c.*) recombinant strains coexpressing *mlbJ* and the ABC transporter genes to NAI-107.
A. *Streptomyces coelicolor* WT, containing the empty vector pRM4, expressing *mlbJ*, *mlbYZ* or coexpressing *mlbJ* and *mlbYZ*.
B. *Streptomyces coelicolor* WT, expressing *mlbTU*, *mlbTU* and *mlbJ*, *mlbEF* or *mlbEF* and *mlbJ*. NAI-107 gradient plates (0–2 μg ml^{-1}).

with the transmembrane protein MlbJ. The stop codon of *mlbJ* overlaps with the start codon of *mlbY* suggesting an operon organization of the genes *mlbJ*, *mlbY* and *mlbZ*. *mlbJ* was cloned into the integrative plasmid pIJ10257 (hyg^R) under the control of the constitutive promoter *ermE*p*. pIJ10257 containing *mlbJ* was transferred to *S. coelicolor* harbouring one of the recombinant plasmids pRM4-*mlbYZ*, pRM4-*mlbTU* and pRM4-*mlbEF* (apra^R) respectively. The recombinant *S. coelicolor* strains were tested against NAI-107 to determine a possible synergistic effect of the expressed proteins.

MlbJ conferred resistance only in the coexpression with MlbYZ (Fig. 4). The cooperative action of MlbJ and MlbYZ suggested a role of MlbJ as substrate-binding protein. The expression of MlbJ alone conferred a low resistance to NAI-107, probably by intercepting the lantibiotic from the extracellular space. MlbJ contains several predicted membrane-spanning regions (SOSUI ver. 1.11; Hirokawa *et al.*, 1998). According to its hydrophobicity profile and function, we propose that MlbJ is functionally analogous to the LanH family of proteins. LanH proteins are accessory proteins that act with the immunity ABC transporters LanFEG (Okuda and Sonomoto, 2011). One of the best-characterized LanH proteins is NukH, a transmembrane protein from *Staphylococcus warneri*. NukH recognizes the lantibiotic nukacin ISK-1 and transfers it to the ABC transporter NukFEG, which then pumps the lantibiotic to the extracellular space in an energy-dependent manner (Okuda *et al.*, 2008). The coexpression of MlbJ with MlbTU and MlbEF did not confer NAI-107 resistance. In the microbisporin cluster, the genes encoding the ABC transporter MibTU are co-transcribed together with the genes *mibBCDTUV* (Foulston and Bibb, 2010). Since the gene organization in the *mlb* gene cluster is identical, the same transcriptional unit can be supposed. Therefore, a role of MlbTU as exporter was speculated. The deletion of the *mlbEF* homologous gene *mibEF* from the microbisporicin gene cluster resulted in markedly delayed and greatly reduced levels of microbisporicin production. Therefore, the authors speculated that MibEF is either involved in immunity or in lantibiotic export (Foulston and Bibb, 2010).

The lipoprotein MlbQ confers resistance specifically to NAI-107-like lantibiotics

Since it is known that lipoproteins often act as substrate binding proteins of ABC transporters in Gram-positive bacteria, MlbQ was tested in coexpression experiments with the ABC transporters encoded in the *mlb* cluster, as reported for the coexpression of *mlbJ*. The resistance assays against NAI-107 did not show any difference between *S. coelicolor* M145 expressing an ABC transporter alone or in combination with MlbQ (data not shown). The coexpression with the ABC transporters did not increase the immunity effect of MlbQ. Therefore, we assumed an MlbQ mode of action independent of the ABC transporters MlbYZ, MlbTU and MlbEF.

To explore the specificity of MlbQ-mediated resistance, *S. coelicolor* pRM4-*mlbQ* was tested against the lantibiotic 97518. 97518, also called planosporicin, is an NAI-107-like lantibiotic that has an identical arrangement of thioether rings to NAI-107 and a similar antibacterial spectrum, but is considerably less potent than NAI-107 (Maffioli *et al.*, 2009). Recently, the elucidation of the 3D structure of NAI-107 and 97518 in solution by NMR underlined the conformational differences between the two related lantibiotics, which might be responsible for the significant difference in potency (Vasile *et al.*, 2011). MlbQ cross-resistance to 97518 was determined by filter disc assay. *S. coelicolor* pRM4-*mlbQ* was grown on a Luria–Bertani (LB) agar plate where a sterile filter disc soaked with 20 μg of 97518 was applied. *Streptomyces coelicolor* pRM4-*mlbQ* was more resistant against 97518 than the WT (Fig. 5A). The 97518 biosynthetic gene cluster encodes a lipoprotein, named PlnQ (GenBank KM588197), which displays 62% amino acid identity to MlbQ. To further investigate the cross-resistance to the lantibiotics NAI-107 and 97518, PlnQ was expressed in *S. coelicolor* M145, and resistance

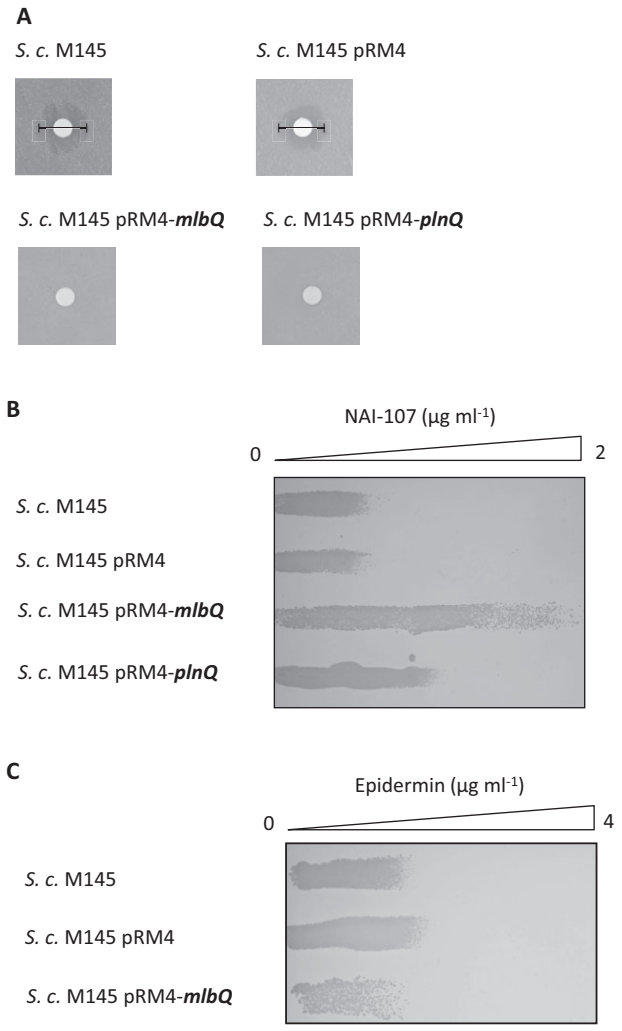

Fig. 5. Resistance of *S. coelicolor* M145 (*S. c.*) recombinant strains expressing *mlbQ* and *plnQ* to the lantibiotics 97518, NAI-107 and epidermin.
A. Resistance of *S. coelicolor* WT, containing the empty vector, expressing *mlbQ* or *plnQ* to 97518 (20 μg).
B. Resistance of *S. coelicolor* WT, containing the empty vector, expressing *mlbQ* or *plnQ* to NAI-107 (gradient plate 0–2 μg ml^{-1}).
C. Resistance of *S. coelicolor* WT, containing the empty vector or expressing *mlbQ* to epidermin (gradient plate 0–4 μg ml^{-1}).

assays were performed (see *Experimental procedures*). PlnQ conferred resistance to both NAI-107 and 97518 (Fig. 5A and B), as observed for the lipoprotein MlbQ. However, *S. coelicolor* pRM4-*plnQ* was less resistant against NAI-107 than *S. coelicolor* pRM4-*mlbQ*. Lantibiotic cross-resistance was previously reported for the membrane-associated proteins PepI and EciI encoded in the Pep5 and epicidin 280 lantibiotic clusters from *Staphylococcus epidermidis* 5 and *S. epidermidis* BN280 respectively (Heidrich *et al.*, 1998). In contrast, cross-resistance was not observed for NisI, the lipoprotein encoded in the nisin gene cluster (*nis*). NisI did not confer resistance to subtilin, a lantibiotic with an identical organization of the thioether rings (Stein *et al.*, 2003). To confirm the specificity of MlbQ action against NAI-107-like lantibiotics, we tested *S. coelicolor* M145 pRM4-*mlbQ* against epidermin (Allgaier *et al.*, 1985). Epidermin is a class I lantibiotic which presents an N-terminal nisin-like lipid II binding motif and a C-terminal aminovinyl cysteine. Epidermin differs from NAI-107 and 97518 in the central region where the flexible 12-15 amino acids are substituted in NAI-107 by the ring C. *S. coelicolor* M145 expressing MlbQ was not more resistant to epidermin than the WT (Fig. 5C). The resistance assays against 97518 and epidermin showed the specificity of MlbQ to NAI-107-like lantibiotics.

MlbQ possesses a hydrophobic surface patch proposed to bind NAI-107

As MlbQ recognizes structurally related lantibiotics and no effect was observed against other antibiotics with the same target, we hypothesized a mode of action based on the direct interaction with the lantibiotic. To assess this, we purified an N-terminally His_6-tagged fusion protein from *E. coli* Rosetta 2(DE3) and determined the structure of MlbQ in solution by NMR spectroscopy (PDB 2MVO) (Fig. 6). MlbQ is divided into an unstructured N-terminal region followed by a globular domain (P55-G147). The domain is wedge shaped, consisting of a C-terminal helix (α3) packed at an angle against a four-stranded β-sheet, with a disulphide bond connecting the helix to the sheet at its C-terminal end. This sheet has a simple meander topology, but features an unusual structured β1-β2 loop at the broad end of the wedge, including a short helix (α3) that runs perpendicular to the sheet. A conserved tryptophan residue (W77) anchors the loop into the hydrophobic core of the fold, while a short β-strand forms a bridge with the long loop between β4 and α3. This loop is largely unstructured, but begins with a short helix (α2) that also runs perpendicular to the sheet, but in the opposite direction to that in the β1-β2 loop.

Three-dimensional (3D) structural comparisons using the DALI server (Holm and Rosenström, 2010) found only limited similarity to known folds. The only significant similarity is to the IQ domain of Ras GTPase activating-like proteins (IQGAP2: Z-score 2.5, RMSD 2.5 Å). This similarity is considerable over the β-meander, β1-β2 loop and C-terminal helix and includes an analogous β-bridge. The major differences are that in IQGAP2, the α2 helix is longer and runs in the opposite direction, almost parallel to α3, and the β-bridge forms part of a larger sheet, giving the fold a sandwich-like character. No sequence similarity between representatives of the IQGAP2 fold and MlbQ could be detected using sensitive profile-based methods.

The only other lantibiotic immunity protein of known structure is SpaI from *Bacillus subtilis* (Christ *et al.*, 2012).

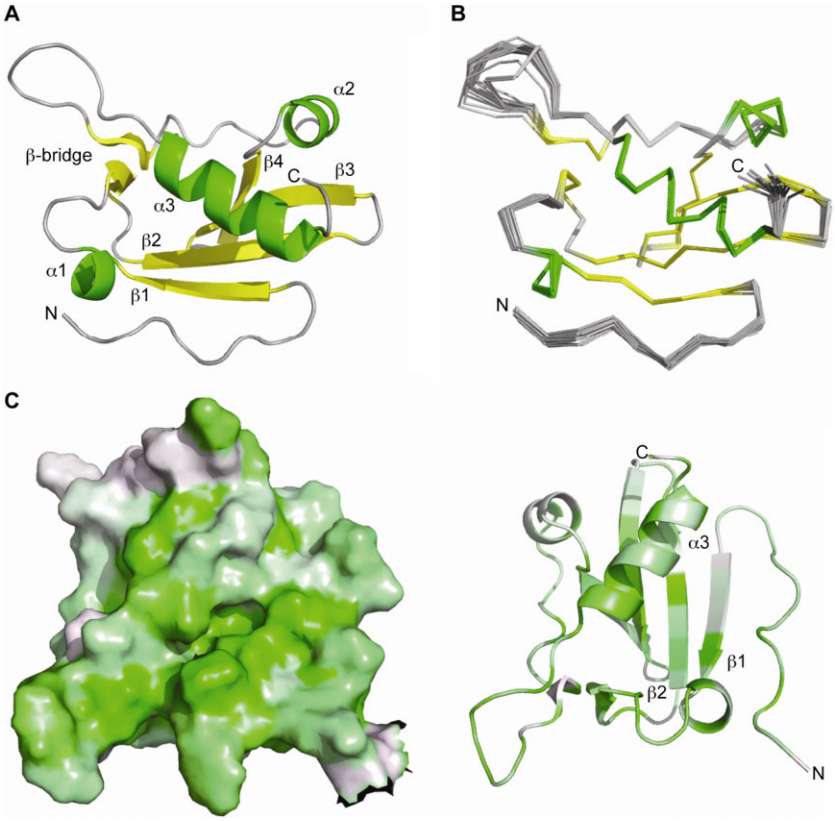

Fig. 6. Solution structure of MlbQ.
A. A cartoon view of the globular domain of the protein (P55-G147). Beta strands are in yellow and helices in green.
B. The 19 structures of the NMR ensemble depicted as a Cα ribbon. The view and secondary structure colouring are as in A. The unstructured N-terminal loop is omitted for clarity. The last segment of the N-terminal loop (P55-N64) runs antiparallel to β1 and is well defined in the ensemble. Details of the superimposition are provided in Table S1.
C. The surface of MlbQ globular domain colored from white to green according to increasing residue hydrophobicity. A distinct hydrophobic patch runs across this face of the protein. The cartoon view on the left shows this patch is bounded by α3, the β1-β2 loop and the structured region of the N-terminal loop.

This protein shows no structural similarity to MlbQ. However, it is proposed to bind its cognate lantibiotic, subtilin, via an extended hydrophobic patch on its surface. Looking for a similar feature on MlbQ, we located an analogous surface patch bounded by the β1-β2 loop, α3, anda structured, conserved hydrophobic section of the N-terminal loop (P55-N64), which runs antiparallel to β1 (Fig. 6). The extended shape of the patch and its length (~16 Å) are comparable to that in SpaI. Unfortunately, the low solubility of the lantibiotic in the NMR buffers precluded mapping of the potential interaction sites for SpaI (Christ *et al.*, 2012), and similar problems were encountered for MlbQ. The extremely poor solubility of the lantibiotic in aqueous buffers also made alternative binding assays with CD or fluorescence spectroscopy impossible (data not shown).

A Microbispora *MlbQ null mutant is strongly impaired in growth in presence of NAI-107*

To determine the effect of MlbQ-mediated immunity in the producer strain, we constructed the markerless deletion mutant Δ*mlbQ* by homologous recombination of the two flanking regions, which were cloned in a non-replicative plasmid. Single and double cross-over events were identified using the GUS reporter system (see *Experimental procedures*). *Microbispora* Δ*mlbQ* was impaired in growth compared with the WT strain from start of NAI-107 production at 48 h (Fig. 7). To exclude that the observed phenotype is due to polar effects caused by the deletion of *mlbQ*, the mutant was complemented *in trans* by expressing *mlbQ* under the control of the constitutive promoter *ermE*p*. The complemented mutant Δ*mlbQ*(pRM4-*mlbQ*) revealed the same phenotype as the WT, demonstrating that the impaired growth of the *mlbQ* mutant is a consequence of *mlbQ* deletion. These observations confirmed that MlbQ is required for normal growth in the presence of NAI-107.

Conclusions

Microbispora ATCC PTA-5024 produces the lantibiotic NAI-107, which interferes with PG biosynthesis by targeting the bactoprenol-pyrophosphate coupled PG precursors. The PG of *Microbispora* exhibits muropeptides consisting of tripeptides, tetrapeptides, pentapeptides and different amino acids in the fourth and fifth position of the tetrapeptides and pentapeptides respectively. The presence of these muropeptides suggests the action of L,D-transpeptidases and D,D-carboxypeptidases in PG biosynthesis and remodelling. Both enzymes were described to play a crucial role in glycopeptide and β-lactam-resistance. Although PG modifications seem not to be responsible for NAI-107 resistance, we can speculate

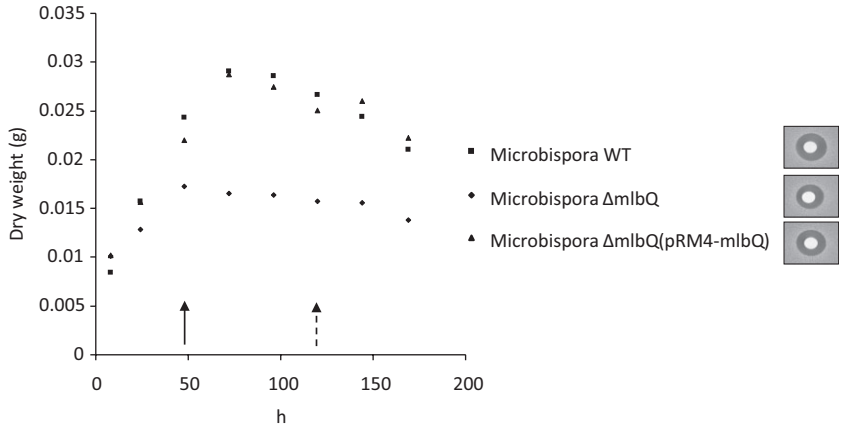

Fig. 7. Growth and NAI-107 production of *Microbispora* WT (■) Δ*mlbQ* (◆) and Δ*mlbQ* (pRM4-*mlbQ*) (▲). NAI-107 production starts at 48 h (black arrow). Samples for the bioassay were taken at 120 h (dashed arrow). The y-axis represents the dry weight of 5 ml samples. $n = 1$, four technical replicates.

a more general role of PG remodelling in antibiotic resistance conferring competitive advantage in the natural environment. However, *Microbispora* possesses NAI-107 specific resistance determinants. The NAI-107 biosynthetic gene cluster (*mlb*) encodes two immunity mechanisms: the MlbJYZ complex and the lipoprotein MlbQ.

We have shown that the ABC transporter MlbYZ confers resistance only together with the transmembrane protein MlbJ, which might act as a substrate-binding protein. The immunity lipoprotein MlbQ acts independently from MlbJYZ. The solution structure of MlbQ revealed an unstructured N-terminal region and a small, globular C-terminal domain. The domain has no similarity in sequence or structure to SpaI, the only lantibiotic-resistance protein of known structure. However, the proteins share a hydrophobic surface patch, which is proposed to bind the cognate lantibiotic.

Experimental procedures

Bacterial strains and plasmids

Escherichia coli XL1-Blue (Bullock *et al*., 1987) was used for cloning experiments, *E. coli* Rosetta 2(DE3) (Novagen) for protein expression, *E. coli* ET12567 pUZ8002 (Kieser *et al*., 2000) and *E. coli* S17-1 (Simon *et al*., 1983) for intergenic conjugation with *S. coelicolor* and *Microbispora* spp. respectively. *Microbispora* JCM 10266 and the NAI-107 producer *Microbispora* ATCC PTA-5024 were obtained from NAICONS (Milan). A strain and plasmid list is given in Table 2.

Media and culture conditions

Escherichia coli strains were grown and manipulated according to Sambrook and Russel, 2001. M9 minimal medium was used for ^{13}C,^{15}N-labeled protein expression in *E. coli* (Chen *et al*., 2006). *Streptomyces coelicolor* was cultivated and genetically manipulated as described in Kieser *et al*., 2000. *Microbispora* spp. were grown in GE82G medium (dextrose monohydrate 20 g l^{-1}, maize dextrin 20 g l^{-1}, soybean meal 15 g l^{-1}, yeast extract 5 g l^{-1}, $CaCO_3$ 1 g l^{-1}, agar 1 g l^{-1}, pH adjusted to 7.3) for the generation of biomass. 10 ml of medium were inoculated with 6% of a glycerol stock in a 100-ml baffled Erlenmeyer flask with a coiled spring baffle and incubated at 30°C and 180 r.p.m. in an orbital shaker. For a larger scale, a 4-day culture was used to inoculate (6% inoculum) 100 ml of medium in a 500-ml baffled Erlenmeyer flask with a coiled spring baffle. *Microbispora* spp. were streaked onto MV0.1 (dextrose monohydrate 0.1 g l^{-1}, maize dextrin 2.4 g l^{-1}, yeast extract 0.5 g l^{-1}, meat extract 0.3 g l^{-1}, tryptose 0.5 g l^{-1}, agar 15 g l^{-1}, pH adjusted to 7.2) and HA (malt extract 10 g l^{-1}, yeast extract 4 g l^{-1}, glucose 4 g l^{-1}, $CaCl_2$ 1.46 g l^{-1}, agar 18 g l^{-1}) agar media. S1 agar medium (oatmeal 60 g l^{-1}, agar 18 g l^{-1}, $FeSO_4 \times 7H_2O$ 1 mg l^{-1}, $MnCl_2 \times 4H_2O$ 1 mg l^{-1}, $ZnSO_4 \times 7H_2O$ 1 mg l^{-1}) was used for sporulation of *Microbispora* spp.

Microbispora *growth curve and NAI-107 susceptibility test*

For the generation of the biomass, *Microbispora* WT and the recombinant strains were grown in GE82G in two pre-cultures of 96 h and 72 h respectively. The second pre-culture was used to inoculate (6% inoculum) 100 ml of Evans medium (glucose 25 g l^{-1}, NaH_2PO_4 10 mM, $NaNO_3$ 50 mM, $FeSO_4 \times 7H_2O$ 0.32 mM, MOPS 21 g l^{-1}, KCl 10 mM, Na_2SO_4 2 mM, citric acid 2 mM, $CaCl_2 \times 2H_2O$ 0.25 mM, $MgCl_2 \times 6H_2O$ 1.25 mM, $NaMoO_4 \times 2H_2O$ 1 μg l^{-1}, $FeCl_3 \times 6H_2O$ 1 mg l^{-1}, $MnCl_2 \times 4H_2O$ 0.05 mg l^{-1}, $ZnCl_2$ 0.2 mg l^{-1}, $CuCl_2 \times 2H_2O$ 0.05 mg l^{-1}, $Na_2B_4O_7 \times 10H_2O$ 0.05 mg l^{-1}, $(NH_4)_6Mo_7O_{24} \times 4H_2O$ 0.05 mg l^{-1}, pH adjusted to 6.5) in a 500-ml Erlenmeyer flask with a coiled spring baffle and incubated at 30°C and 180 r.p.m. in an orbital shaker. For the determination of the dry weight, 5 ml samples were taken at 8 h, 24 h, 48 h, 96 h, 120 h, 144 h, 168 h, centrifuged and lyophilized. The NAI-107 susceptibility test was conducted using *Micrococcus luteus* as tester strain in LB agar (10^5 cfu ml^{-1}). One to three microlitres of the supernatant of *Microbispora* cultures (120 h) were pipetted on sterile paper disks. For *Microbispora* WT always 2 μl were used, whereas for the recombinant strains the volume of the supernatant was adjusted to the dry weight of the cultures. As a reference, the dry weight of the wild type was set as 100%. Sterile paper discs were applied on *M. luteus* plates which were incubated for 24 h at 37°C.

Table 2. List of strains and plasmids.

Strain/plasmid	Description	Source, reference
E. coli XL1 Blue	Cloning host	Bullock *et al.*, 1987
E. coli Rosetta 2(DE3)	Protein expression	Novagen
E. coli ET12567 pUZ8002	Methylation-deficient *E. coli*, conjugation *S. coelicolor* M145	Kieser *et al.*, 2000
E. coli S17-1	Methylation-proficient *E. coli*, conjugation *Microbispora* strains	Simon *et al.*, 1983
Microbispora ATCC PTA-5024	WT, NAI-107 producer	NAICONS
Microbispora RP0	Non-producer, disruption of *mlb* cluster by single cross-over integration of the plasmid pGusA21-*mlbAB*	This study
Microbispora Δ*mlbQ*	*mlbQ* deletion mutant of *Microbispora* ATCC PTA-5024	This study
Microbispora Δ*mlbQ* (pRM4-*mlbQ*)	*mlbQ* deletion mutant complemented with pRM4-*mlbQ*	This study
Microbispora JCM 10266	WT	Nukajima *et al.*, 1999
S. coelicolor M145	$SCP1^-$ $SCP2^-$	Kieser *et al.*, 2000
pGusA21	Non-replicative, *gusA*, $Apra^r$	Muth, unpublished
pGusA21-*mlbAB*	Non-replicative, *gusA*, fragment of *mlbAB*, $Apra^r$	This study
pA18gus	Non-replicative, *gusA*, $Apra^r$	This study
pA18gusΔ*mlbQ*	Non-replicative, *gusA*, upstream and downstream regions of *mlbQ*, $Apra^r$	This study
pRM4	pSET152 derivative, ΦC31 attP, constitutive promoter *ermE**, $Apra^r$	Menges *et al.*, 2007
pRM4-*mlbQ*	pRM4 derivative, *mlbQ*, $Apra^r$	This study
pRM4-*mlbJ*	pRM4 derivative, *mlbJ*, $Apra^r$	This study
pRM4-*mlbYZ*	pRM4 derivative, *mlbYZ*, $Apra^r$	This study
pRM4-*mlbTU*	pRM4 derivative, *mlbTU*, $Apra^r$	This study
pRM4-*mlbEF*	pRM4 derivative, *mlbEF*, $Apra^r$	This study
pIJ10257	ΦBT1 attP, constitutive promoter *ermE**, Hyg^r	John Innes Institute, Norwich
pIJ10257-*mlbQ*	pIJ10257 derivative, *mlbQ*, Hyg^r	This study
pIJ10257-*mlbJ*	pIJ10257 derivative, *mlbJ*, Hyg^r	This study
pET30-*mlbQ*	pET30 derivative, used for the expression of MlbQ (25-129 aa) as a N-terminal His-tag fusion protein in *E. coli*, Kan^r	This study
1G6	Cosmid containing the *mlb* cluster, $Apra^r$	NAICONS, Milan
4B8	Cosmid containing the biosynthetic gene cluster of 97518, $Apra^r$	NAICONS, Milan

Peptidoglycan analysis

Microbispora spp. was grown in KV6 medium (dextrose monohydrate 12 g l^{-1}, soy peptone 12 g l^{-1}, yeast extract 12 g l^{-1}, NaCl 2 g l^{-1}, pH adjusted to 7.5) at 28°C for 72 h (exponential phase), and the PG was isolated as previously described (Schaeberle *et al.*, 2011). Harvested cells were boiled in SDS, washed several times with water and disrupted with a Precellys homogenizer. Cell walls were purified by digestion with 50 μg ml^{-1} RNase, 10 μg ml^{-1} DNase (2 h) and 100 μg ml^{-1} trypsin (18 h) and subsequently treated with 48% hydrofluoric acid (HF) (48 h) to remove teichoic acids. The isolated PG was digested with mutanolysin (18 h), and the released muropeptides were reduced with sodium borohydride. Muropeptides were separated by HPLC on an Agilent 1200 HPLC using a linear gradient of eluent A = 5% MeOH in 100 mM sodium phosphate buffer (pH 2.5) and eluent B = 30% methanol in 100 mM sodium phosphate buffer (pH 2.8) for 150 min (time in min: $t_0 = t_5 = 0\%B$, $t_{150} = 100\%B$, 0.5 ml min^{-1}, 52°C) on a Prontosil 120 C18 column (250 × 4.6 mm, 3 μm, Bischoff Chromatography, Leonberg, Germany). The injection volume was 100 μl. Muropeptides were detected at 205 nm. High-performance liquid chromatography–mass spectrometry analyses of muropeptide samples were performed with an Agilent HPLC-ESI-MS system (LC/MSD Ultra Trap System XCT 6330, Waldbronn, Germany), using a linear gradient of eluent A = 0.1% formic acid in water and eluent B = 0.06% formic acid in methanol (time in min: $t_0 = t_5 = 5\%B$, $t_{155} = t_{180} = 30\%B$, 0.5 ml min^{-1}, 52°C) on a Reprosil Gold 300 C18 column (250 × 4.6 mm ID, 5 μm, Dr. Maisch, Ammerbuch). The injection volume was 90 μl. Detection of *m/z* values was conducted with AGILENT DATAANALYSIS for 6300 Series Ion Trap LC/MS 6.1 ver. 3.4 software (Bruker-Daltonik). MS^2 and MS^3 data were acquired with electrospray ionization in the positive mode. MS^3 spectra (Fig. S1) were annotated using the software MMASS (Niedermeyer and Strohalm, 2012).

Heterologous expression of putative immunity genes in S. coelicolor *M145*

mlbQ, mlbYZ, mlbTU, mlbEF, mlbJ and *plnQ* were amplified from the cosmids 1G6 (*Microbispora* ATCC PTA-5024) and 4B8 (*Planomonospora* DSM 14920) with the primers listed in Table 3 to yield 5′ NdeI and 3′ EcoRI sites, cloned into pJet1.2 (Fermentas) and confirmed by sequencing. The genes were subsequently cloned into pRM4 downstream the constitutive promoter *ermE*p*, and the respective plasmids were integrated at the φC31 site of *S. coelicolor* M145. Ex-conjugants were selected with 50 μg ml^{-1} apramycin and confirmed by colony PCR.

Coexpression of MlbQ and MlbJ with the ABC transporters MlbYZ, MlbTU, MlbEF in S. coelicolor *M145*

mlbQ and *mlbJ* were amplified from the cosmid 1G6 (*Microbispora* ATCC PTA-5024) with the primers mlbQfor1/mlbQrev2 and mlbJfor1/mlbJrev2 (Table 3) and cloned into pJet1.2 (Fermentas). Error-free clones were digested with NdeI/HindIII and cloned into pIJ10257 under the control of the constitutive promoter *ermE*p* yielding pIJ10257-*mlbQ* and pIJ10257-*mlbJ*. pIJ10257-*mlbQ* or pIJ10257-*mlbJ* was inte-

Table 3. List of primers.

Primer	Sequence (5′-3′)	Experiment, restriction sites
mlbAfor	AAGCTTCCGGGCCGGCGGTGACGAGCTGG	Disruption *mlb* cluster, HindIII
mlbBrev	CATATGCGGTGTCTCGCTCGGCGGCCACTTG	Disruption *mlb* cluster, NdeI
mlbQfor1	ACATATGACGAACACGACCAGAGCCCGCCTGTCC	*mlbQ* cloning in pRM4 and pIJ10257, NdeI
mlbQrev1	TAGAATTCCGTGTTCGTCATTCAGCCCTTTCCGCAGAG	*mlbQ* cloning in pRM4, EcoRI
mlbQrev2	ATAAGCTTTCAGCCCTTTCCGCAGAG	*mlbQ* cloning in pIJ10257, HindIII
mlbQupfor	GAATTCTCGCCGCGGTCGCCGAGGG	Deletion *mlbQ*, amplification upstream region *mlbQ*, EcoRI
mlbQuprev	GGATCCGCCCTGTACGTAGCGTCCGGCCACCC	Deletion *mlbQ*, amplification upstream region *mlbQ*, BamHI
mlbQdownfor	GGATCCGACCAGATCCGGGACCACAGG	Deletion *mlbQ*, amplification downstream region *mlbQ*, BamHI
mlbQdownrev	AAGCTTCCGGTGCGACTACGGGGATGC	Deletion *mlbQ*, amplification downstream region *mlbQ*, HindIII
mlbQhistagfor	GACGACGACAAGACGGGCGGCGGCAGAG	MlbQ expression in *E. coli* Rosetta 2(DE3), Ek/LIC cloning site
mlbQhistagrev	GAGGAGAAGCCCGGTTCAGCCCTTTCCGCAGAG	MlbQ expression in *E. coli* Rosetta 2(DE3), Ek/LIC cloning site
plnQfor	CATATGACCGGCGAACACCGGCGTCC	*plnQ* cloning in pRM4, NdeI
plnQrev	ATGAATTCTCATCGGGCGAACCGGCAGAGC	*plnQ* cloning in pRM4, EcoRI
mlbYZfor	ATCATATGACAGGCAGGTGGCTGGTCCCGCTCACG	*mlbYZ* cloning in pRM4, NdeI
mlbYZrev	GAATTCATTCATGGCCGCGGACCCGTGG	*mlbYZ* cloning in pRM4, EcoRI
mlbTUfor	ATCATATGACGGTCCCGGCGTTCGAGCTCAG	*mlbTU* cloning in pRM4, NdeI
mlbTUrev	GAATTCTCACCTCCCCACCCGCAGCCGCAG	*mlbTU* cloning in pRM4, EcoRI
mlbEFfor	ATCATATGGCGGCCCTGATATCCAC	*mlbEF* cloning in pRM4, NdeI
mlbEFrev	GAATTCTCATCTCACTGCGGAACCC	*mlbEF* cloning in pRM4, EcoRI
mlbJfor1	CATATGGAGATGGTCTCTGTCGAGTTG	*mlbJ* cloning in pRM4 and pIJ10257, NdeI
mlbJrev1	ATGAATTCTCATACCGCCTCTCCC	*mlbJ* cloning in pRM4, EcoRI
mlbJrev2	ATAAGCTTTCATACCGCCTCTCCC	*mlbJ* cloning in pIJ10257, HindIII

grated in the φBT1 site of *S. coelicolor* M145 recombinant strains harbouring one of the recombinant plasmids pRM4-*mlbYZ*, pRM4-*mlbTU*, pRM4-*mlbEF* to obtain *S. coelicolor* M145 strains coexpressing MlbQ or MlbJ with each ABC transporter. Ex-conjugants were selected with 50 μg ml^{-1} apramycin, 50 μg ml^{-1} hygromycin and confirmed by colony PCR.

Manipulation of Microbispora *ATCC PTA-5024 and* Microbispora *JCM 10266*

A protocol for conjugation between the DNA methylation proficient *E. coli* S17-1 and *Microbispora* spp. was established. An aliquot (1 ml) of *Microbispora* glycerol stock was inoculated in 10 ml of nutrient broth medium (Difco). After 20 h, the culture was sonicated for 20 min in a water bath sonicator, the mycelium recovered by centrifugation at 4270 *g* for 10 min, washed once with the medium and concentrated 1:10. *E. coli* S17-1 was grown overnight, washed twice with LB and re-suspended in 1/10 of the initial volume. 100 μl of *Microbispora* mycelium were mixed with 100 μl of *E. coli*, and the mixture was plated on MS medium containing 10 mM $MgCl_2$ (Kieser *et al*., 2000). After 24 h of incubation at 30°C, each plate was overlaid with 1 ml water containing 1500 μg apramycin and 1250 μg phosphomycin. After about 10 days of incubation at 30°C, ex-conjugants were streaked onto HA plates containing 25 μg ml^{-1} apramycin and 50 μg ml^{-1} phosphomycin and checked by colony PCR.

Construction of Microbispora *recombinant strains*

To obtain the non-producer *Microbispora* RP0 strain, the *mlb* cluster was disrupted by single cross-over integration of the pGusA21-*mlbAB* plasmid containing the region from 34 672 bp to 36 222 bp (GenBank: AWEV00000000.1), which comprises part of the genes *mlbA* and *mlbB* encoding the precursor peptide and the dehydratase respectively (primers mlbAfor and mlbBrev, Table 3). The deletion mutant of *mlbQ* was generated by double cross-over of the plasmid pA18gusΔ*mlbQ* containing a 1509 bp upstream fragment (EcoRI/BamHI) and a 1513 bp downstream fragment (BamHI/HindIII) of *mlbQ*. The deletion vector pA18gusΔ*mlbQ* was introduced into *E. coli* S17-1 by transformation and then conjugated into *Microbispora*. The integration of pA18gusΔ*mlbQ* by a single cross-over (apramycin resistant) was identified by GUS agar plate-based assay (Myronovskyi *et al*., 2011). Briefly, *Microbispora* recombinant clones were streaked onto MV0.1 agar plates and after 7 days incubation at 30°C overlaid with 1 mg ml^{-1} X-Gluc (Sigma). A Δ*mlbQ* mutant was selected by screening colonies for the second cross-over (apramycin sensitive), and the correct gene deletion was confirmed by PCR.

Lantibiotic resistance bioassay

The resistance of *S. coelicolor* recombinant strains to the lantibiotics NAI-107 and epidermin was tested using the gradient plate technique (Szybalski and Bryson, 1952). Gradient plates (0–2 or 0–4 μg ml^{-1}) were prepared as described below. Two layers of agar were poured successively into a squared plate (10 × 10 mm) to obtain a gradient from one side to the other. The desired volume of lantibiotic (final concentration of 2 or 4 μg ml^{-1}) was added to 30 ml LB agar, which was poured into the plate. The plate was propped up using a 3 mm thick bar, just enough for the agar to cover the entire bottom. In this way, the agar formed a wedge that was shallow on one side and deep on the other side of the plate. After agar solidification, the plate was placed in a horizontal position, and 30 ml LB agar without lantibiotic was poured to have a homogenous agar layer. After incubation at room temperature to allow lantibiotic diffusion (1–2 h), 2×10^5

spores of *S. coelicolor* strains were spotted at the edges of the gradient plate and streaked to the centre. The plates were incubated 2 days at 30°C.

Since the lantibiotic 97518 is not as potent as NAI-107 and epidermin, resistance assays using gradient plates could not be performed. The 97518 resistance assay was performed by plating out 10^6 spores on an LB agar plate and placing sterile paper discs soaked with 20 μg 97518. Plates were photographed after 2 days incubation at 30°C. NAI-107 and 97518 (NAICONS) were dissolved in 10% dimethylsulphoxide to a final concentration of 1 mg ml^{-1}. Epidermin, isolated from *S. epidermidis* Tü 3298 (Hoerner *et al*., 1989), was dissolved in 0.05% acetic acid.

MlbQ purification

MlbQ without the signal peptide (25–129 aa) was expressed in *E. coli* as an N-terminally His_6-tagged fusion protein. MlbQ was amplified with the primers mlbQhistagfor and mlbQhistagrev (Table 3) and cloned into the vector pET30 Ek/LIC (Novagen) yielding pET30-*mlbQ*. *Escherichia coli* Rosetta 2(DE3) was transformed with the plasmid pET30-*mlbQ* and cultivated in 2 L ^{13}C,^{15}N-labeled M9 minimal medium (BioExpress, Cambridge Isotope Laboratories) supplemented with 30 μg ml^{-1} kanamycin and 25 μg ml^{-1} chloramphenicol at 37°C. Protein expression was induced by addition of 0.5 mM IPTG at an $OD_{600\,nm}$ of 0.6. After 16 h cultivation at 18°C, cultures were harvested and the pellet re-suspended in 30 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, 20 mM $MgCl_2$, pH 8) supplemented with 1 mg ml^{-1} lysozyme, 0.1 mg ml^{-1} DNase and protease inhibitors (Roche). Cells were disrupted by a French Pressure Cell Press (SIM 3 AMINCO Spectronic Instruments, 3 × 1000 Psi) and after centrifugation (47 800 *g*, 30 min, 4°C), the soluble fraction was used for protein purification by Ni-NTA affinity chromatography (Ni-NTA Sepharose Column, 5 ml, IBA). The supernatant was loaded on the pre-equilibrated Ni-NTA column, the matrix washed two times with 20 ml wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM Imidazole, pH 8) and eluted six times with 2 ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM Imidazole, pH 8). Fractions were checked for the presence of the desired protein on 15% SDS-PAGE and visualized by Coomassie blue staining. Most of the protein was eluted in fractions 3, 4 and 5, which were collected together and dialysed against the storage buffer (50 mM NaH_2PO_4, 50 mM NaCl, pH 7.8). After dialysis the protein was further purified by anion exchange chromatography using an ÄKTA purifier system and a MonoQ H55/5 column (GE Healthcare) with a linear salt gradient (Buffer A: 20 mM Tris/HCl, pH 8 and Buffer B: 20 mM Tris/HCl, 1 M NaCl, pH 8). The protein eluted at ~250 mM NaCl, was collected and concentrated with 10 000 MWCO Amicon Ultra centrifugal filter devices (Millipore). The protein concentration was determined using Bradford assay.

NMR structure determination

All spectra were recorded at 298 K on Bruker AVIII-600 and AVIII-800 spectrometers. Backbone sequential assignments were completed using a strategy based on a 3D-HN(CA)NNH experiment (Weisemann *et al*., 1993). Aliphatic side-chain assignments were completed with standard TOCSY-based experiments, while aromatic assignments were made by linking aromatic spin systems to the respective C^bH_2 protons in a 2D-NOESY spectrum. Stereospecific assignments and the resulting C1 rotamer assignments were determined from a combination of HNHB and HA[HBHN](CACO)NH (Löhr *et al*., 1999) experiments. Distance data were derived from a set of five 3D-NOESY spectra, including the heteronuclear edited NNH-, CCH- and CNH-NOESY spectra (Diercks *et al*., 1999) in addition to conventional ^{15}N- and ^{13}C-HSQC-NOESY spectra. A ^{12}C-filtered 2D-NOESY spectrum was recorded for the observation of contacts to aromatic groups. Backbone dihedral angle restraints were derived using the TALOS+ server (Shen *et al*., 2009). Generic backbone dihedral restraints designed to restrict residues to allowed regions of the Ramachandran map and well-populated side-chain rotamers were applied for unstructured residues. Hydrogen bond restraints were applied as pseudo-covalent bonds, as outlined in Truffault and colleagues (2001). Refinement was carried out by comparing experimental and back-calculated NOESY spectra using in-house software. Strips were back-calculated for the amide protons of all ordered atoms, plus selected side-chain groups. These were compared with the experimental spectra to confirm backbone and side-chain dihedral angles and to extract additional distance restraints. Structures were calculated with XPLOR (NIH version 2.9.4) using standard protocols with modifications for the inclusion of H-bonds as pseudo-covalent bonds. For the final set, 100 structures were calculated and 18 chosen on the basis of lowest restraint violations. An average structure was calculated and regularized to give a structure representative of the ensemble. Details of the input data and the final ensemble are given in Table S1.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Mass spectrometry analysis of the monosaccharide tetrapeptides from *Microbispora* ATCC PTA-5024.
A. Structure and fragmentation pattern of the reduced monosaccharide tetrapeptides with 724.5 *m/z* (R:-H) and 738.5 *m/z*

(R:-CH_3). MS^3 spectrum of the reduced monosaccharide tetrapeptides (B) 724.5 *m/z* (parental ion 927.4 *m/z*) and (C) 738.5 *m/z* (parental ion 941.4 *m/z*) in the positive mode. The different fragments between the tetrapeptide monomers MurNAc-L-Ala-D-γ-iGln-*meso*-A_2pm-Gly and MurNAc-L-Ala-D-γ-iGln-meso-A_2pm-D-Ala are underlined. M: molecular ion, b-ions: N-terminal fragments, y-ions: C-terminal fragments.

Fig. S2. High-performance liquid chromatography separation of the muropeptides from *Microbispora* ATCC PTA-5024 and *Microbispora* RP0.

Table S1. Solution structure statistics.