A relA-dependent regulatory cascade for auto-induction of microbisporicin production in Microbispora corallina

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Summary

Microbisporicin is a potent type I lantibiotic produced by the rare actinomycete Microbispora corallina that is in preclinical trials for the treatment of infections caused by methicillin-resistant isolates of Staphylococcus aureus (MRSA). Analysis of the gene cluster for the biosynthesis of microbisporicin, which contains two unique post-translationally modified residues (5-chlorotryptophan and 3, 4-dihydroxyproline), has revealed an unusual regulatory mechanism that involves a pathway-specific extracytoplasmic function sigma factor (MibX)/anti-sigma factor (MibW) complex and an additional transcriptional regulator MibR. A model for the regulation of microbisporicin biosynthesis derived from transcriptional, mutational and quantitative reverse transcription polymerase chain reaction analyses suggests that MibR, which contains a C-terminal DNA-binding domain found in the LuxR family of transcriptional activators, functions as an essential master regulator to trigger microbisporicin production while MibX and MibW induce feed-forward biosynthesis and producer immunity. Moreover, we demonstrate that initial expression of mibR, and thus microbisporicin production, is dependent on the ppGpp synthetase gene (relA) of M. corallina. In addition, we show that constitutive expression of either of the two positively acting regulatory genes, mibR or mibX, leads to precocious and enhanced microbisporicin production.

Introduction

Lantibiotics are ribosomally synthesised, post-translationally modified peptides with antimicrobial activity that are produced by a number of Gram-positive bacteria

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Antibiotic production in actinomycetes is triggered frequently by nutrient limitation (Bibb, 2005; Martín and Lirias, 2012) presumably affording a selective advantage to the producing organism under starvation conditions. Guanosine tetraphosphate (ppGpp) is a key intracellular signalling molecule for sensing nutrient starvation and triggering adaptive responses in a wide range of bacteria. ppGpp induces a rapid response to amino acid starvation in Escherichia coli, Streptomyces species and other bacteria, reducing the expression of genes involved in rapid growth and often activating transcription of genes involved in specialised metabolism (Takano and Bibb, 1994; Bremer and Ehrenberg, 1995; Ochi, 2007; Gaca et al., 2015). Under conditions of nitrogen limitation, the ribosome-bound RelA synthesises ppGpp in response to uncharged tRNAs that bind to the ribosomal A site (Cashel, 2004). ppGpp regulation of gene expression appears to be mediated through GTP, therefore modulating promoter activity indirectly (Krásný and Gourse, 2004). In E. coli, ppGpp elicits transcriptional changes by interacting directly with RNA polymerase (Magnusson et al., 2005) while in Bacillus subtilis, ppGpp regulation of gene expression appears to be mediated through GTP pool levels, therefore modulating promoter activity indirectly (Krásný and Gourse, 2004). In Streptomyces coelicolor, ppGpp synthesis was shown to be required for antibiotic production under conditions of nitrogen limitation (Chakraburtty and Bibb, 1997); moreover, induction of ppGpp synthesis at levels that did not influence growth rate and under conditions of nutritional sufficiency invoked the transcription of the pathway-specific regulatory gene actII-orf4 and actinorhodin production (Hesketh et al., 2001).

In this study, we demonstrate the role of RelA, and presumably ppGpp synthesis, in the activation of microbisporicin biosynthesis by initially triggering the production of a precursor that subsequently induces high levels of production of the mature antibiotic. We also show that the lantibiotic can act as an extracellular signalling molecule to trigger microbisporicin production in the wider M. corallina community. We identify the individual targets of MibX and MibR, firmly establishing the complex regulatory cascade that leads to microbisporicin biosynthesis.

Finally, we identify an ABC transporter that appears to confer some level of immunity to microbisporicin, and that is also required for production of the lantibiotic.

Results

gusA transcriptional fusions in S. coelicolor M1152 verify the targets of MibX and MibR

Microbisporicin production occurs in a growth phase-dependent manner, commencing towards the end of rapid growth (Foulston, 2010). The microbisporicin biosynthetic gene cluster consists of six operons (Fig. 1A). Previous studies (Foulston and Bibb, 2011) identified an ECF-sigma factor consensus sequence (GAACC-N15-GCTAC) located 8–10 nucleotides upstream of the transcriptional start sites of mibJ, mibQ, mibR, mibX and mibE, suggesting that the transcription of these genes and operons is activated directly by MibX. In contrast, the promoter region of the crucial mibABCDTUUV biosynthetic operon lacks this sequence, but contains instead the motif TTGACA-N17-TCGACT that is likely to be recognised by the RNA polymerase holoenzyme containing the major vegetative sigma factor of M. corallina [the homologue of sigma hrdB of S. coelicolor (Brown et al., 1992; Foulston and Bibb, 2011)]. MibR, which is essential for microbisporicin production, could thus be a candidate for activating the transcription of the mibA operon in a growth phase-dependent manner. To evaluate these bioinformatic predictions, the mibJ (299bp), mibQ (166 bp), mibR (384 bp), mibX (254 bp), mibA (244 bp) and mibE (440 bp) promoter regions were cloned separately upstream of a Streptomyces codon-optimised β-glucuronidase gene, gusA, in pGUS (Myronovskiy et al., 2011), and the resulting plasmids (Table 1) integrated into the φC31 attB sites of S. coelicolor M1152 derivatives M1598, M1594 and M1595; the last two strains carried derivatives of pJ10257 (which integrates at the φBT1 attB site) in which mibR or mibX, respectively, were constitutively expressed from ermE′p, while M1598 contained just the vector pJ10257 (Table 1).

Only very low levels of gusA expression were detected from the six mib promoters in the absence of mibR and mibX (Fig. 1B). However, constitutive expression of mibX resulted in marked induction of GusA activity from the mibJ, mibQ, mibR, mibX and mibE promoters, all of which contain the ECF-sigma factor consensus sequence, but not from the mibA promoter fusion in which the ECF-sigma factor motif is absent (Fig. 1B, induction indicated by black stars). In contrast, induction of the mibA promoter was only detected in the strain containing the constitutively expressed mibR (Fig. 1B, indicated by the white star). Thus, in S. coelicolor, MibR activates transcription of the mibA operon whereas MibX activates transcription of the other five genes and operons in the microbisporicin biosynthetic cluster.

Analysis of the mibR promoter region reveals a second promoter independent of MibX

The studies described earlier demonstrated that the ECF-like mibR promoter (mibRp1) is indeed dependent on the ECF-sigma factor MibX for its activation. However, earlier S1 nuclease protection analyses had suggested that there was a second mibR promoter located further upstream (Foulston and Bibb, 2011) that might also be involved in the activating mibR expression. In an attempt to further characterise this putative promoter, reverse transcription polymerase chain reaction (RT-PCR) experiments

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Fig. 1. A. The microbisporicin biosynthetic gene cluster (Foulston and Bibb, 2010). Transcriptional start sites are marked by arrows and those promoter regions containing the predicted ECF-sigma factor consensus sequence are indicated by stars; the loop downstream of mibA indicates a putative attenuator. B. β-glucuronidase activity assays of mib promoter regions fused to gusA in Streptomyces coelicolor M1152 and derivatives containing constitutively expressed mibR (R++) or mibX (X++) were carried out after 72 h of growth. β-Glucuronidase activity is expressed as Miller units mg\(^{-1}\) of protein. The filled stars indicate promoters regulated by MibX and the open star indicates the promoter regulated by MibR.

### Table 1. Plasmids used and constructed in this study.

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pUZ8002 | tra, neo, RP4 | J. Wilson and D. Figurski, personal communication |
| pIJ8600 | oriT, ΦC31 attB-int, APR\(^{\circ}\), tipAp | Takano et al., 1995 |
| pIJ12572 | pIJ8600 + mibR | This work |
| pIJ10257 | oriT, ΦBT1 attB-int, HYG\(^{\circ}\), ermE\(^{\circ}\) | Hong et al., 2005 |
| pIJ12574 | pIJ10257 + mibX | This work |
| pIJ12576 | pIJ10257 + mibR | This work |
| pIJ12743 | pIJ10257 + mibEF | This work |
| pIJ12750 | pIJ10257 + I-SceI | This work |
| pIJ12738 | pKC1132 with MCS and I-SceI site from pUC57-Simple_SceI | Fernández-Martínez and Bibb, 2014 |
| pGUS | oriT, ΦC31 attB-int, APR\(^{\circ}\), promoterless codon-optimised gusA | Myronovskiy et al., 2011 |
| pIJ12579 | pGUS + mibA promoter region | This work |
| pIJ12580 | pGUS + mibE promoter region | This work |
| pIJ12581 | pGUS + mibJ promoter region | This work |
| pIJ12582 | pGUS + mibQ promoter region | This work |
| pIJ12583 | pGUS + mibR whole (including p1 and p2) promoter region | This work |
| pIJ12584 | pGUS + mibX promoter region | This work |
| pIJ12586 | pGUS + mibR\(^{\circ}\) promoter region | This work |

APR, apramycin; HYG, hygromycin B.
Transcriptional activation of mibRp2 is relA-dependent

Previous studies had implicated ppGpp, produced by the ribosome-bound ppGpp synthetase (RelA), as an intracellular signalling molecule for the initiation of antibiotic production in actinomycetes (Takano and Bibb, 1994; Martínez-Costa et al., 1998; Ochi, 2007). Moreover, deletion of relA in S. coelicolor abolished the production of both actinorhodin and the undecylprodigine complex of compounds under conditions of nitrogen limitation (Chakraburty and Bibb, 1997), while induction of ppGpp synthesis at levels that did not impair growth activated actinorhodin gene transcription (Hesketh et al., 2001).

To determine whether transcription from mibRp2 was dependent on ppGpp, a 478 bp fragment containing mibRp2 (corresponding to nt sequence 5245–5723 of GenBank accession HM536998.1) was cloned in pGUS generating pIJ12586, which was then introduced into the ΦC31 attB sites of S. coelicolor M145 (relA+) and S. coelicolor M571 (ΔrelA), and GusA activity assayed throughout growth in nitrogen-limited Supplemented liquid Minimal Medium (SMM) (Fig. 3A). The results demonstrate that transcription from mibRp2 in S. coelicolor under these growth conditions is RelA- (and presumably ppGpp) dependent.

Deletion of relA in M. corallina abolishes microbisporicin production

To determine whether microbisporicin production was RelA-dependent in the natural producer, we adopted the Meganuclease strategy (Fernández-Martínez and Bibb, 2014) to construct a markerless relA deletion mutant of M. corallina (see Experimental Procedures). M. corallina is a difficult strain to work with; it grows slowly, taking 2–3 weeks to obtain workable colonies and it sporulates poorly, necessitating the use of mycelial fragments for most genetic manipulations. Consequently, the availability of the Meganuclease system that allows the selection of second crossover events during gene deletion by homologous recombination is extremely valuable. The resulting
A model for the regulation of microbisporicin production in M. corallina

Based on the results presented so far, we propose an update to our previous model (Foulston and Bibb, 2011) for the regulation of microbisporicin production that explains its growth phase-dependence (Fig. 4). During growth under conditions of nutrient sufficiency, the system is poised for activation with MibX, produced from a basal level of expression, sequestered at the membrane by its cognate anti-sigma factor MibW (mibXW are co-transcribed) (Foulston and Bibb, 2010; 2011). Under conditions of nitrogen limitation, uncharged tRNAs bind to the ribosomal A-site, activating the ribosome-bound RelA resulting in ppGpp synthesis. This then leads to a low level of expression of mibR from the relA-dependent mibRp2 promoter, and consequently, a low level of transcription of the mibABCDDTUV operon and the production of a small amount of the immature and less active form of the lantibiotic lacking the chlorination of tryptophan at position 4, and the di-hydroxylation of proline at position 14 (Lazzarini et al., 2005) This less active form of the lantibiotic is exported out of the cell by the ABC transporter MibTU (Foulston and Bibb, 2010) where it could interact either with MibW or its target lipid II resulting in cell envelope stress potentially sensed by RelA; the likely subsequent ppGpp synthesis then leads to a feed-forward regulatory mechanism that results in high levels of lantibiotic biosynthesis.

Constitutive expression of mibR or mibX triggers early and enhanced production of microbisporicin

The data and model presented thus far indicate that both MibR and MibX function as transcriptional activators to trigger microbisporicin biosynthesis. To assess the effect of constitutive expression of mibX and mibR on microbisporicin production, pJ12572, containing mibR under the control of the thiostrepton-inducible tipA promoter and plJ12574, containing mibX under the control of the constitutive ermE' promoter, were integrated into the ΦC31 and φBT1 attB sites of M. corallina, respectively. Constitutive expression of mibR (the tipA promoter exhibits basal levels of expression in the absence of thiostrepton; Murakami et al., 1989; Ali et al., 2002) resulted in precocious and increased levels of microbisporicin production.
which were further enhanced in the presence of the inducer (Fig. 5A; confirmed by Matrix-Assisted Laser Desorption/Ionisation-Time of Flight (MALDI-ToF) analysis, data not shown). Constitutive expression of mibX also caused precocious microbisporicin production and at much higher levels than in the wild-type strain (Fig. 5B). Simultaneous constitutive expression of both mibR and mibX resulted in even higher levels of microbisporicin biosynthesis (Fig. 5B). These results confirm that both MibR and MibX function as positively acting regulators of microbisporicin biosynthesis in *M. corallina*.

**Microbisporicin acts as a signalling molecule in**

*M. corallina to induce its own production*

The model presented earlier for the regulation of microbisporicin production is essentially an example of autoinduction, where the production of a small amount of the
immature form of the lantibiotic functions in a feed-forward mechanism initiated by nutrient limitation and ppGpp synthesis to subsequently trigger high levels of production. But in principle, once transported out of the cell, both forms of the lantibiotic could interact with other members of the \textit{M. corallina} community that are not nutrient limited to coordinate microbisporicin production, perhaps in an attempt to achieve ecologically relevant levels of antibiotic activity. To address this possibility, \textit{M. corallina} M1592 (the mibR over-expression strain), which produces microbisporicin precociously, was spotted on V0.1 agar (Marcone \textit{et al}., 2010) plates in close proximity to streaks of the wild-type \textit{M. corallina} strain and the \(\Delta \text{mibA}\) non-producing mutant M1127 (Foulston and Bibb, 2010). After 5 days of incubation, when the wild-type strain had not commenced microbisporicin biosynthesis (usually detected from 6 days onwards), the plates were overlaid with soft nutrient agar containing the indicator strain \textit{Micrococcus luteus} (Fig. 6 left plate). The inverted pear-shaped zone of inhibition revealed induction of microbisporicin biosynthesis in the wild-type mycelia closest to M1592 while the inhibition zone next to the non-producer control strain remained circular. To confirm that this induction of production was due to microbisporicin, 2.5 µg of the lantibiotic were spotted onto an antibiotic assay disc next to streaks of wild-type \textit{M. corallina} and the \(\Delta \text{mibA}\) non-producing mutant. Again, the inverted pear-shaped inhibition zone indicated autoinduction of microbisporicin production (Fig. 6, right plate) in the wild-type strain, but not in the \(\text{mibA}\) mutant. A range of other antibiotics was also tested for their ability to induce microbisporicin production, including the cell wall biosynthesis inhibitors vancomycin, planosporicin, fosfomycin, bacitra-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{A. Antimicrobial activity of M1592 (\textit{Microbispora corallina} containing mibR expressed from tipAp) compared with the wild-type strain (WT). The strains were grown in VSPA at 30°C and culture supernatants sampled at different time points. M1592 was also grown at two different concentrations of thiostrepton (inducer of tipAp). Forty microlitre of samples of culture supernatants were applied to filter paper discs, which were dried and placed on a lawn of \textit{Micrococcus luteus}. The plate was incubated overnight at 30°C before zones of inhibition were visualised. B. Antimicrobial activity of \textit{M. corallina} constitutively expressing mibR (M1592), mibX (M1593) and both mibR and mibX simultaneously (M1597) compared with the wild-type strain (WT). Strains were grown in VSPA liquid medium at 30°C and 40 µl of samples of culture supernatants were applied to filter paper discs which were dried and placed on a lawn of \textit{Micrococcus luteus}. The plate was incubated overnight at 30°C before zones of inhibition were visualised.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{Microbisporicin induces its own production. Left plate: \textit{Microbispora corallina} M1592 constitutively expressing mibR and precociously producing the lantibiotic was spotted next to streaks of (left) the wild-type (WT) strain and (right) a non-producing \(\Delta \text{mibA}\) mutant. Right plate: Addition of purified microbisporicin to a filter paper disc triggers precocious microbisporicin biosynthesis in the \textit{M. corallina} wild-type strain, but not in the \(\Delta \text{mibA}\) mutant. The strains were grown on MV01 agar medium at 30°C for 5 days and then overlaid with a lawn of \textit{Micrococcus luteus} in soft nutrient agar. The plates were incubated overnight at 30°C before zones of inhibition were visualised. The inverted pear-shaped inhibition zones indicate precocious induction of microbisporicin production in the mycelium located proximal to the source of the compound.}
\end{figure}
cin, carbenicillin and tunicamycin and the protein synthesis inhibitor apramycin (data not shown). A range of concentrations were used, but none induced precocious microbisporicin production even at concentrations at which some of the antibiotics inhibited growth of *M. corallina*. These results demonstrate that microbisporicin can induce its own synthesis at subinhibitory concentrations and in a highly specific manner.

*MibEF are likely to be involved in immunity to microbisporicin in *M. corallina*.*

Previous bioinformatic analysis of the *mib* gene cluster identified three pairs of genes encoding ABC transporters: *mibTU*, *mibEF* and *mibYZ*. While deletion of *mibTU* had no apparent effect on microbisporicin production, the latter was essentially abolished in a *mibEF* mutant (Foulston and Bibb, 2010). To assess the possible role of MibEF in immunity, wild-type *M. corallina* and the Δ*mibEF* mutant (M1131) were grown on V0.1 agar medium containing increasing concentrations of microbisporicin (Fig. 7A). The wild-type strain grew well on 0.75 \( \mu \text{g} \text{ ml}^{-1} \) of microbisporicin, whereas the Δ*mibEF* mutant failed to grow at 0.025 \( \mu \text{g} \text{ ml}^{-1} \) indicating an approximate 30-fold increase in sensitivity to the compound. B. *Streptomyces coelicolor* M145 containing pJ10257 (empty vector) or pJ12743 (with mibEF expressed constitutively from ermE*') were grown on R5 agar medium containing increasing concentrations of microbisporicin. Expression of mibEF resulted in an approximate twofold reduction in sensitivity to microbisporicin.

**Discussion**

In this study, we demonstrate that RelA, and presumably ppGpp synthesis, can activate the complex regulatory pathway that leads to the biosynthesis of microbisporicin,
a potent lantibiotic currently undergoing preclinical trials. Our model suggests that an immature and less active form of microbisporicin serves as the initial extracellular signalling molecule to coordinate production throughout the *M. corallina* population. This may reflect a mechanism adopted by the organism to protect itself and its siblings from the highly potent mature lantibiotic, ensuring expression of *mibTU* before committing to the production of microbisporicin. A similar feed-forward regulatory mechanism has been proposed for the lantibiotic planosporicin (Sherwood and Bibb, 2013). Interestingly, in this case, planosporicin, which is considerably less active than microbisporicin (Sherwood and Bibb, 2013), serves as the specific signalling molecule.

Auto-induction of antibiotic biosynthesis has also been observed in low-GC Gram-positive lantibiotic producing bacteria; examples include nisin (Kleerebezem, 2004), subtilin (Stein et al., 2002) and mersacidin (Schmitz et al., 2006), where at least some of the compounds have been suggested to function as quorum sensors that monitor population size and density (Stein et al., 2002; Kleerebezem, 2004). In contrast, in *M. corallina* and potentially other actinomycetes, auto-induction is triggered by nutrient limitation which we propose serves to coordinate production of the antibiotic throughout the mycelial population, not all of which may be experiencing starvation, with the aim of achieving ecologically effective levels of the antibiotic.

We have shown that deletion of *mibEF* in *M. corallina* results in increased sensitivity to microbisporicin. Furthermore, heterologous over-expression of *mibEF* in *S. coelicolor* resulted in an increase in resistance to the lantibiotic, suggesting that MibEF plays a role in conferring immunity to microbisporicin in the natural producer. In previous work, deletion of *mibEF* essentially abolished microbisporicin production (Foulston and Bibb, 2010). The closest homologues of MibEF (with the exception of transporters encoded by closely related actinobacterial lantibiotic gene clusters, such as that for planosporicin; Sherwood and Bibb, 2013) are the immunity ABC transporters found in low-GC Gram-positive lantibiotic producers. This raises the intriguing possibility of the existence, at least for microbisporicin, of a fail-safe mechanism that ensures that the production of a potent antibiotic does not take place unless the corresponding immunity system is in place. How this potential mechanism influences the expression of *mibR* and/or *mibX* and hence, microbisporicin biosynthesis remains to be determined.

Preclinical trials suggest that microbisporicin is a promising candidate for clinical development, which will require the provision of significant amounts of the purified compound. In addition to deciphering the complex regulatory mechanism that triggers microbisporicin production, we have demonstrated, by constitutively expressing the genes encoding the two transcriptional activators present in the *mib* gene cluster, how that knowledge can be used to markedly increase the productivity of the wild-type strain, hopefully contributing to the future clinical use of this compound.

The experiments reported here on *M. corallina* were also carried out on the commercial producer of microbisporicin (NAI-107) *Microspora* sp. ATCC PTA5024 with essentially the same results. For continuity with previous work (Foulston and Bibb, 2010; 2011), only the *M. corallina* results are reported here.

**Experimental procedures**

*Strains and general methods*

The strains used and generated in this study are listed in Table 2. *E. coli* strains were grown and manipulated following standard methods (Sambrook et al., 1989; Gust et al., 2003). For conjugation, *M. corallina* NRRL 30420 spores were germinated for 21 h in 10 ml Difco Nutrient Broth (DNB, Becton Dickinson, Sparks, Maryland, USA), resuspended in 500 µl DNB, mixed with *E. coli* S17 (Simon et al., 1983) carrying the relevant conjugative and integrative vector and plated on Soya Flour Mannitol (SFM) agar containing 10 mM MgCl2. After growth at 30°C for 20 h, the plates were overlaid with 100 µl of fosfomycin (100 mg ml⁻¹) and the appropriate concentration of the antibiotic used to select for the vector. Plates were grown at 30°C until putative exconjugants were visible (3–5 weeks). Microbisporicin was detected as described previously (Foulston and Bibb, 2010). *S. coelicolor* strains were grown and manipulated as described previously (Kieser et al., 2000). Plasmids and oligonucleotides are described in Tables 1 and S1, respectively.

**Construction of a relA mutant of M. corallina**

Chromosomal regions flanking the *relA* coding sequence (Sosio et al., 2014) were amplified by PCR and cloned into pIJ12738 (Fernández-Martínez and Bibb, 2014). The 5’ flanking region was amplified to generate a 1902 bp fragment with terminal *Ncol* and *EcoRI* sites while the 3’ region was amplified to generate a 1945 bp fragment with terminal *EcoRI* and *Xbal* sites. These two fragments were cloned into pIJ12738 digested with *Ncol* and *Xbal* to generate pIJ12749 with the I-Scel site adjacent to the introduced fragments. pIJ12749 was then introduced into *E. coli* S17 by transformation. Conjugation between the *E. coli* S17 derivative and *M. corallina* was carried out as described earlier. Chromosomal integration of pIJ12749, confirmed by PCR analysis (data not shown), generated *M. corallina*. The 806 bp *Ndel*-SacII fragment containing the I-Scel Meganuclease gene codon-optimised for expression in actinomycetes was excised from pIJ12739 (Fernández-Martínez and Bibb, 2014) and cloned into pIJ10257 to generate pIJ12750, which was then conjugated into *M. corallina*. A 10 µg ml⁻¹ and grown at 30°C until sporulation.
PCR analysis showed that one of these exconjugants, M1596, lacked the chromosomal relA sequence (data not shown).

gusA transcriptional fusions

Promoter regions from the mib gene cluster (P_mib, 299 bp, P_mib, 166 bp, P_mib, 384 bp, P_mib, 254 bp, P_mib, 244 bp and P_mib, 440 bp) were amplified using oligonucleotides with 5′ XbaI and 3′ KpnI sites, confirmed by nucleotide sequencing and ligated into pGUS (Myronovskyi et al., 2011) digested with the same enzymes to generate the following plasmids containing gusA under the control of each of the promoters: pIJ12579 (P_mib-gusA), pIJ12580 (P_mib-gusA), pIJ12581 (P_mib-gusA), pIJ12582 (P_mib-gusA), pIJ12583 (P_mib-gusA) and pIJ12584 (P_mib-gusA). The plasmids were integrated at the fC31 attB site of S. coelicolor M1152 (Gomez-Escribano and Bibb, 2011) after conjugation and at the same site in S. coelicolor M1594 and M1595, strains carrying constructs based on pIJ10257 (Hong et al., 2005) in which mibR or mibX, respectively, were expressed constitutively from ermE promoter. Exconjugants were selected using apramycin (25 μg ml⁻¹) and confirmed by PCR.

β-Glucuronidase assays

Spectrophotometric β-glucuronidase assays were carried out as described previously (Sherwood and Bibb, 2013). Enzymatic activity was plotted as Miller units calculated as 1000 × (OD420 of sample – OD420 of blank)/[time of reaction in minutes × volume of culture assayed (in ml)] and expressed per milligram of protein.

Constitutive expression of mibR and mibX in M. corallina and S. coelicolor M1152

PCR products containing mibR or mibX (extending from start to stop codons) were generated by high-fidelity PCR using the primers listed in Table S1 and confirmed by nucleotide sequencing. The mibR fragment was cloned into the integrative vector pJL8600 (Takano et al., 1995) to fuse mibR to the inducible tipA promoter generating pIJ12572, which was introduced into M. corallina by conjugation. For introduction into S. coelicolor M1152, the same mibR fragment was cloned into the integrative vector pJL10257 to fuse mibR to the constitutive ermE′ promoter generating pIJ12576. Similarly, the mibX

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**Table 2.** Strains used and constructed in this study.

| Strain                  | Genotype                                                                 | Reference                                |
|-------------------------|--------------------------------------------------------------------------|------------------------------------------|
| *Escherichia coli* DH5α  | F− ϕ80 lacZ/M15 ∆(lacZYA-argF)U169 recA1 endA1 hsdR17 (K′, mcrA)          | Invitrogen™                               |
| E. coli ET12567         | dam-13:: Tnθ dcm-6 hsdM GHL, carrying helper plasmid pUZ8002              | MacNeil et al., 1992                     |
| E. coli S17             | E. coli strain carrying an integrated RP4 derivative                      | Simon et al., 1983                       |
| *M. corallina* NRRL 30420 | *M. corallina* wild-type strain                                          | Nakajima et al., 1999                    |
| *M. corallina* M1592    | *M. corallina* + pIJ12572                                                | This work                                |
| *M. corallina* M1593    | *M. corallina* + pIJ12574                                                | This work                                |
| *M. corallina* M1597    | *M. corallina* + pIJ12572 + pIJ12574                                     | This work                                |
| *M. corallina* M1596    | *M. corallina* + M1596                                                   | This work                                |
| *M. corallina* M1127    | *M. corallina*, mibA::aac(3)IV                                           | Foulston and Bibb, 2010                  |
| *M. corallina* M1131    | *M. corallina*, mibE::aac(3)IV                                           | Foulston and Bibb, 2010                  |
| *M. corallina* M571     | *M. corallina* + M571                                                    | This work                                |
| *S. coelicolor* M1152   | M1152 + pIJ10257                                                        | This work                                |
| *S. coelicolor* M1598   | M1598 + pIJ12576                                                        | This work                                |
| *S. coelicolor* M1594   | M1594 + pIJ12576                                                        | This work                                |
| *S. coelicolor* M1595   | M1595 + pIJ12576                                                        | This work                                |
| *S. coelicolor* M1594 derivatives | M1594 + pIJ12576, M1594 + pIJ12580, M1594 + pIJ12581, M1594 + pIJ12582, M1594 + pIJ12583, M1594 + pIJ12584 | This work                                |
| *S. coelicolor* M1595 derivatives | M1595 + pIJ12579, M1595 + pIJ12580, M1595 + pIJ12581, M1595 + pIJ12582, M1595 + pIJ12583, M1595 + pIJ12584 | This work                                |

CHL, chloramphenicol.
fragment was also cloned into the integrative vector pJ10257 to fuse mibX to the constitutive ermE′ promoter generating pJ12574. These constructs were transferred into *M. corallina* by conjugation from *E. coli* S17 and into *S. coelicolor* M1152 by conjugation from *E. coli* ET12567/pUZ8002 as described previously (Kieser *et al.*, 2000).

**Expression of mibEF in *S. coelicolor* M145**

A PCR product containing mibEF (extending from the start codon of mibE to the stop codon of mibF) was generated by high-fidelity PCR using the primers listed in Table S1 and confirmed by nucleotide sequencing (data not shown). This fragment was cloned into the integrative vector pJ10257 to fuse mibEF to the constitutive ermE′ promoter generating pJ12743. This plasmid was transferred into *S. coelicolor* M145 by conjugation from *E. coli* ET12567/pUZ8002 as described previously (Kieser *et al.*, 2000).

**RT-PCR analysis**

The *M. corallina* wild-type strain was grown in 10 ml VSP liquid medium (Marcone *et al.*, 2010) until an OD_{600} of 0.4 was reached (after 3–4 days of growth) and then 1 ml of this culture was transferred to 50 ml VSP liquid medium (designated time 0). For nested RT-PCR analysis of the mibR promoter (Fig. 2), RNA was extracted from the mycelia from 4 ml of culture sampled after 16, 24, 40 and 48 h of growth from time 0 (Tunca *et al.*, 2007). For RT-PCR analysis of the effect of deletion of relA on mib gene expression, *M. corallina* wild-type and the relA mutant strain were grown in 10 ml SMM medium (Kieser *et al.*, 2000) to an OD_{600} of 0.4 (reached after 6–7 days of growth) and then 1 ml of this culture was transferred to 50 ml SMM liquid medium (designated time 0). RNA was extracted from the mycelia from 4 ml of culture sampled after 48 h of growth from time 0 (Tunca *et al.*, 2007). Mycelial pellets were resuspended in 1 ml RTL buffer with lysing matrix B (MP Biomedicals, Solon, Ohio, USA) and homogenised using a Camlab Omni Bead Ruptor 24 Drive Unit (Camlab, Cambridge, UK). Two pulses of 30 s of intensity 6.0 were applied with cooling down for 1 min on ice between pulses. Supernatants were centrifuged for 10 min at 13,000 r.p.m. and then treated according to the instructions given in the RNA Easy Kit (Qiagen, Crawley, UK). The RNA samples were treated with DNase I (Invitrogen) until they were free of DNA contamination (determined by PCR amplification). RNA was quantified and equal amounts from each sample were converted to cDNA following the manufacturer’s instructions (SuperScript®, Invitrogen). Oligonucleotide pairs listed in Table S1 were used to amplify nested fragments of the mibR promoter region. The oligonucleotide pairs used to analyse expression of the mib cluster in the wild-type and relA mutant strains were described previously (Foulston and Bibb, 2010). Amplification was also attempted using the same oligonucleotide pairs on RNA samples that had not been treated with reverse transcriptase to confirm lack of DNA contamination.

**RACE**

The 5′ end of the mibRp2 transcript was identified by using a 5′ RACE (rapid amplification of cDNA ends) kit (Invitrogen, Paisley, UK, version 2.0) following the manufacturer’s instructions. Briefly, first-strand cDNA synthesis was carried out using 5 μg of RNA, reverse transcriptase and the oligonucleotide primer RACE_mibR_R1 (Table S1). cDNA was purified using the SNAP columns provided in the kit, and poly(dC) tails were added to the 3′ ends using terminal deoxynucleotidyl transferase. PCR amplification of the tailed cDNA was carried out initially using the 5′ RACE abridged anchor primer with the first-strand primer RACE_mibR_R1 (Table S1). A dilution of the PCR mixture was then subjected to a second amplification using the abridged anchor primer with the second nested primer RACE_mibR_R2 (Table S1). The PCR product was gel-purified and a portion sequenced directly using oligonucleotide RACE_mibR_R2 as primer (RACE1 in Fig. S1B). Another portion was used for cloning into pGEM- TEasy (Promega UK, Southampton, UK) and the cloned PCR fragment sequenced using RACE_mibR_R2 as primer (RACE2 in Fig. S1B).

**Induction of microbisporicin production in *M. corallina***

To test for induction of microbisporicin production in wild-type *M. corallina*, either *M. corallina* M1592 (mib expression strain) or 2.5 μg of purified microbisporicin (in 10% DMSO) dried on a filter paper disc were placed on V0.1 (Marcone *et al.*, 2010) agar plates adjacent to streaks of wild-type *M. corallina* and a non-producing mutant [ΔmibA, (Foulston and Bibb, 2010)] as a control; the plates were overlaid with *Micrococcus luteus* after incubation at 30°C for 5 days.

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Supporting information

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