Production of the antibiotic FR-008/candicidin in *Streptomyces* sp. FR-008 is co-regulated by two regulators, FscRI and FscRIV, from different transcription factor families

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In *Streptomyces* sp. FR-008, the biosynthetic gene cluster of the polyene antibiotic FR-008, also known as candicidin, consists of 21 genes, including four regulatory genes, *fscRI*–*fscRIV*. Our bioinformatics analyses indicate that FscRI has an N-terminal PAS domain, whereas the other three regulators have N-terminal AAA domains and are members of the LAL (large ATP-binding regulators of the LuxR type) family. Deletion of *fscRI* abolished the production of FR-008, with production restored in the complemented strain, supporting a critical role for FscRI in FR-008 biosynthesis. Consistent with these findings, transcription of genes involved in the biosynthesis and efflux of FR-008 was greatly downregulated in a Δ*fscRI* mutant. Interestingly, the regulatory gene *fscRIV* was also downregulated in the Δ*fscRI* mutant. Production of FR-008 was reduced, but not abrogated, in an *fscRIV* deletion mutant, and although structural genes were downregulated in Δ*fscRIV*, the changes were much less dramatic than in Δ*fscRI*, suggesting a stronger regulatory role for FscRI. Remarkably, transcription of *fscRI* was also decreased in Δ*fscRIV*. Expression of *fscRI* restored antibiotic production in a Δ*fscRIV* mutant, but not vice versa. Putative binding sequences for FscRI were identified upstream of *fscRIV* and the three structural genes *fscA*, *fscB* and *fscD*, which encode large modular polyketide synthases. Our findings suggest that *fscRI* and *fscRIV* are interregulatory, whereas expression of *fscRII* and *fscRIII* appears to be independent of *fscRI* and *fscRIV*. This study demonstrates that the regulation of polyene antibiotic synthesis can involve mutually regulated transcriptional activators that belong to different families.

**INTRODUCTION**

*Streptomyces* produce a great variety of secondary metabolites that account for more than half of medically important antimicrobial and anti-tumour agents (Hopwood, 2007), including the antifungal polyene macrolides (Zotchev, 2003). Polyene antibiotics comprise a family of type I polyketide macrolide ring compounds with backbones of 20–40 or more carbons, containing several conjugated double bonds (Gupte et al., 2002; Zotchev, 2003). Their antifungal activities result mostly from specific binding to ergosterol, a major component of the fungal membrane, causing the formation of channels that lead to the leakage of cellular materials (Bolard, 1986).

Recently, gene clusters responsible for the biosynthesis of polyene antibiotics, including nystatin, amphotericin, pimaricin, candicidin and FR-008, have been cloned and sequenced (Aparicio et al., 2000, 2003; Brautaset et al., 2000; Caffrey et al., 2001; Campelo & Gil, 2002; Chen et al., 2003; Gil & Campelo-Diez, 2003), and multiple regulatory genes were revealed in almost all of the clusters. Two of these, PimM and PimR, are positive, and pathway-specific, regulators for pimaricin production in *Streptomyces natalensis* (Antón et al., 2004, 2007; Santos-Arberturas et al., 2011a, b), and are the most well-studied regulators associated with polyene antibiotic biosynthesis. PimR contains an N-terminal SARP (*Streptomyces* antibiotic regulatory protein) domain and a C-terminal half that is homologous to LAL regulators. PimR-like

*One supplementary table and six supplementary figures are available with the online Supplementary Material.*

†These authors contributed equally to this work.

**Abbreviations:** EMSA, electrophoresis mobility shift assay; HTH, helix–turn–helix; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR; TSP, transcriptional start point.

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regulators include PteR of the pentaene filipin pathway in *Streptomyces avermitilis* (Ikeda et al., 2003), SanG of the nikkomycin pathway in *Streptomyces anschromogenes* (Liu et al., 2005) and PoR of the polyoxin pathway in *Streptomyces cacaoi* (Li et al., 2009).

PimM has an N-terminal PAS sensory domain and a C-terminal helix–turn–helix (HTH) motif for DNA binding. PAS domains were named for their homology to the *Drosophila* period protein (Per), the aryl hydrocarbon receptor nuclear translocation protein (Arnt) and the *Drosophila* single-minded protein (Sim) (Taylor & Zhulin, 1999). Proteins with a PAS domain are generally believed to be able to sense changes in redox potential, oxygen, light, the overall energy level of a cell and small ligands (Taylor & Zhulin, 1999). Regulators similar to PimM are light, the overall energy level of a cell and small ligands to be able to sense changes in redox potential, oxygen, single-minded protein (Sim) (Taylor & Zhulin, 1999), receptor nuclear translocation protein (Arnt) and the period protein (Per), the aryl hydrocarbon receptor nuclear translocation protein (Arnt) and the period protein (Per) (Taylor & Zhulin, 1999). The consensus sequence required for PimM binding has been identified and PimM binding sites were identified upstream of several structural genes responsible for the initiation and first elongation cycles of polyketide chain extension (Santos-Aberturas et al., 2011a, b).

Besides PimM-like and PimR-like regulators, members of the LAL (large ATP-binding regulators of the LuxR type) family of regulatory proteins were recently reported to regulate the biosynthesis of macrolide antibiotics. PikD, the only regulator in the pikromycin cluster, positively regulates the production of pikromycin in *Streptomyces venezuelae* (Wilson et al., 2001). In some cases, multiple LAL-type regulators are present in the same cluster, especially in polyene antibiotic biosynthetic clusters, such as NysRI–NysRIII of the nystatin cluster in *S. noursei* ATCC 11455 (Sekurova et al., 2004), AmphRIV of the amphotericin pathway in *Streptomyces nodosus* (Carmody et al., 2004), NysRI of the nystatin cluster in *Streptomyces noursei* (Sekurova et al., 2004), SalRIII of the salinomycin cluster in *Streptomyces noursei* (Taylor & Zhulin, 1999). Regulators similar to PimM are present in several polyene antibiotic pathways, including AmphRIV of the amphotericin pathway in *Streptomyces nodosus* (Carmody et al., 2004), NysRIV of the nystatin cluster in *Streptomyces noursei* (Sekurova et al., 2004), SalRIII of the salinomycin cluster in *Streptomyces albus* (Knischová et al., 2007) and ScNRII of the natamycin pathway in *Streptomyces chattanoogensis* (Du et al., 2009). The consensus sequence required for PimM binding has been identified and PimM binding sites were identified upstream of several structural genes responsible for the initiation and first elongation cycles of polyketide chain extension (Santos-Aberturas et al., 2011a, b).

**METHODS**

**Bacterial strains and culture conditions.** The wild-type *Streptomyces* sp. FR-008 and its derivative strains (Table 1) were grown at 30 °C on SFM agar for spor production or in YEME medium for mycelium preparation (Kieser et al., 2000). *Escherichia coli* Novablue and RosettaBL21(DE3)pLYsS (Novagen) were cultivated in Luria-Bertani (LB) liquid medium and were used for general cloning and protein expression, respectively. *E. coli* ET101E was used for conjugation between *E. coli* and *Streptomyces*. Ampicillin (100 µg ml⁻¹), ampramycin (50 µg ml⁻¹), thiostrepton (20 µg ml⁻¹), kanamycin (25 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹) and nalidixic acid (25 µg ml⁻¹) were added, as appropriate, to growth medium for selection of transformants either from *E. coli* or *Streptomyces*.

**RNA isolation, reverse transcription PCR (RT-PCR) and real-time PCR.** *Streptomyces* sp. FR-008 and its derivative strains were cultivated for 48 h in YEME (supplemented with 10.3 % sucrose) at 30 °C with shaking (220 r.p.m.) before being collected by centrifugation (5000 r.p.m./10 min). Total RNA was extracted using an RNA extraction kit (SBSBIO) and processed as recommended by the manufacturer. RT-PCR and real-time PCR were carried out as described by Zhang et al. (2014). Real-time PCR assays were performed using a Roche LightCycler480 thermal cycler, and relative quantities of cDNA were normalized for the hrrB gene, which encodes the major sigma factor in *Streptomyces*. Sequences of primers used in this study are listed in Table S1 (available in the online Supplementary Material).

**RNA ligase-mediated rapid amplification of cDNA ends (RACE).** *Streptomyces* sp. FR-008 total RNA was extracted and processed using the FirstChoice RLM-RACE kit (Ambion), as described (Zhang et al., 2014). The 5′ RACE Outer primer (Ambion) and FscRI Outer primer were used for Outer PCR. For Inner PCR, the 5′ RACE Inner primer (Ambion) was paired with the FscRI Inner primer for Inner PCR. PCR products were analysed on an agarose gel (1.5 %) using the 100 bp DNA ladder (TransGen Biotech) as size marker. Specific bands were excised, purified and cloned into pGEM-T-Easy (Promega). Ten clones were sequenced to determine the transcriptional start point (TSP).

**In-frame deletions of fscRI and fscRIV in Streptomyces sp. FR-008.** To delete fscRI from *Streptomyces* sp. FR-008, we used a strategy similar to that used for deleting SHJG8833 from *Streptomyces hygroscopicus* 5008 (Zhang et al., 2014). First, the deletion plasmid, pFscRI-MU, was constructed using left-arm and right-arm fragments that flank the deleted region, as follows. The 1805 bp DNA left-arm fragment was amplified by PCR using primers FscRI-Left arm-forward (with a HindIII adaptor) and FscRI-Left arm-reverse (with an Xhol adaptor). Similarly, the 1086 bp right-arm fragment was obtained using primers FscRI-Right arm-forward (with an Xhol adaptor) and FscRI-Right arm-reverse (with an Xhol adaptor). The two fragments were ligated separately into pCR-Blunt (Invitrogen) to
generate pLeft-Arm and pRight-Arm. Next, pLeft-Arm was cut by XhoI and XbaI (present in the multiple cloning site of pCR-Blunt) and ligated to the right arm excised by XhoI and XbaI from pRight-Arm, generating pRL-Arm, which is missing 240 bp of fscRI. The fused arms were removed by HindIII and XbaI digestion, and ligated into pTU1278, which was predigested with HindIII and XbaI, to obtain the deletion construct pFscRI-MU.

To generate ΔfscRI, pFscRI-MU was transformed into E. coli ET12567 containing pUZ8002 and was then introduced into Streptomyces sp. FR-008 by intergeneric conjugation, as described by Kieser et al. (2000). Exconjugants were screened for thioestrepton resistance and were then plated on non-selective solid MS medium (Kieser et al., 2000). Exconjugants were screened for thiostrepton resistance and thiostrepton complementation at various stages of conjugation. Exconjugants were then screened for thiostrepton resistance and thiostrepton complementation at various stages of conjugation. Exconjugants with resistance to apramycin were screened, confirmed by PCR analysis, and named ΔfscRI-Com and ΔfscRIV-Com, respectively.

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td><strong>Streptomyces</strong></td>
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<td>FR-008</td>
<td>Wild-type strain that produces FR-008</td>
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<td>ΔfscRI</td>
<td>Has in-frame deletion of 243 bp from fscRI</td>
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<tr>
<td>ΔfscRI-Com</td>
<td>ΔfscRI complemented with pRI-Com</td>
<td>This study</td>
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<tr>
<td>ΔfscRI-Com-II</td>
<td>ΔfscRI complemented with pRIV-Com</td>
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<tr>
<td>ΔfscRI-ComCK</td>
<td>ΔfscRI with pSET152</td>
<td>This study</td>
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<tr>
<td>ΔfscRIV</td>
<td>Has in-frame deletion of 2559 bp from fscRIV</td>
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<td>ΔfscRIV-Com</td>
<td>ΔfscRIV complemented with pRIV-Com</td>
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<td>Novahoe</td>
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<td>Strain used for conjugation between E. coli and Streptomyces species</td>
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<td>Streptomyces integrative vector</td>
<td>Kieser et al. (2000)</td>
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<td>This study</td>
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<td>pSET152 containing the coding sequence of fscRI plus 558 bp of its upstream sequence</td>
<td>This study</td>
</tr>
<tr>
<td>pRIV-Com</td>
<td>pSET152 containing the coding sequence of fscRIV plus 206 bp of its upstream sequence</td>
<td>This study</td>
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Construction of pRI-Com and pRIV-Com. To generate a complemented strain, the coding sequence of fscRI, plus all the upstream intergenic region (558 bp), was amplified by PCR using primers RIC-Com-forward/reverse and RIC-Com-forward/reverse and then inserted into pMD18-T. Following sequencing verification, the insert was released and ligated with the pre-cut integrating plasmid pSET152 to generate pRI-Com. pRIV-Com was constructed by inserting the coding sequence of fscRIV together with 206 bp of its upstream region, amplified by RIVCom-forward/reverse, into pSET152. All plasmid constructs were verified by sequencing in BioSun.

pRI-Com and pRIV-Com were introduced, respectively, into ΔfscRI and ΔfscRIV to generate complemented strains through conjugation. Exconjugants with resistance to apramycin were screened, confirmed by PCR analysis, and named ΔfscRI-Com and ΔfscRIV-Com, respectively.

Construction of an fscRI expression plasmid and purification of FscRI. The coding sequence of fscRI was amplified using primers FscRI express-Forward (with an Ndel adaptor) and FscRI expressReverse (with an XhoI adaptor), and the PCR products were cloned into pMD18-T (Takara). Following sequence verification, fscRI inserts were excised by Ndel and XhoI digestion, gel-purified and ligated into Ndel/XhoI-cut pET15b (Invitrogen), an expression vector containing an N-terminal His tag and thrombin site, to generate pExp-FscRI. The expression plasmid was used to transform E. coli RosettaBL21(DE3)pLysS (Novagen). Expression of fscRI was induced by the addition of IPTG (1.5 mM) with incubation for 4 h at 28 °C. Bacteria were collected by centrifugation at 4 °C and were sonicated in binding buffer [50 mM Tris/HCl (pH 8.0), 200 mM NaCl, 20 mM imidazole]. Lysates were centrifuged for 10 min at 13 000 r.p.m., and tagged FscRI was recovered from the supernatant using Ni-NTA resin (Bio Basic). The resin was washed twice with washing buffer [50 mM Tris/HCl (pH 8.0), 200 mM NaCl, 40 mM imidazole] before the tagged protein was eluted with elution buffer [50 mM Tris/HCl (pH 8.0), 200 mM NaCl, 200 mM imidazole]. The purified proteins (Fig. S3) were then dialysed in dialysis cassettes [10 000 Da molecular weight cutoff; Thermo Scientific] against a buffer composed of 50 mM Tris/HCl (pH 8.0), 50 mM NaCl and 5% glycerol, then concentrated using a centrifugal filter (10 000 Da molecular weight cutoff; Millipore). Protein concentration was determined by performing a bicinchoninic acid assay (Pierce).
Electrophoresis mobility shift assays (EMSAs). DNA fragments were amplified and labelled at the 3' end with biotin-11-UTP using the Biotin 3' End DNA Labelling kit (Thermo Scientific), according to the manufacturer's instruction. EMSA reactions were performed as described by Zhang et al. (2014). For competition assays, an excess of unlabelled competitor DNA was included. Reaction mixtures were analysed on non-denaturing polyacrylamide gels. After electrophoresis, the probe DNA was transferred from the gel to nylon membrane, fixed by baking for 20 min at 120 °C, and then blocked by incubating with blocking buffer (0.12 M NaCl, 0.017 M Na2HPO4, 0.008 M NaH2PO4, 0.17 M SDS) for 30 min at room temperature. After blocking, the nylon membrane was further incubated in fresh blocking buffer containing horseradish peroxidase-labelled streptavidin (Boehringer) for 20 min, and then washed twice in washing buffer I (a 1:10 dilution of blocking buffer) and washing buffer II (0.01 M Tris-base, 0.002 M MgCl2, pH 9.5), before being developed using the ECL Western blotting Analysis System (GE Healthcare) and exposed to radiographic film (Kodak).

Bioassay and HPLC analysis. The wild-type strain FR-008 and its derivatives were grown on SFM agar medium for 4 days, and a patch of the agar medium was then transferred to a layer of agar seeded with Rhodotorula rubra, which was obtained from the China General Microbiological Culture Collection Center, to test for growth inhibition of this indicator fungus by bioassay. The FR-008 complex produced by Streptomyces sp. FR-008 and its mutant strains was extracted and detected as described by Chen et al. (2003). HPLC was performed using a Hypersil ODS (C18) 5 µ column (Thermo Scientific) with UV detection at a flow rate of 1 ml min⁻¹ on a Shimadzu LC-10AT system. Solvent A was 0.15 % (v/v) aqueous formic acid and solvent B was acetonitrile. Samples were detected using a gradient of 15–90 % solvent B in 35 min.

RESULTS

Bioinformatics analysis of fscRI, fscRII, fscRIII and fscRIV

Four consecutive regulatory genes, fscRI, fscRII, fscRIII and fscRIV, were annotated in the FR-008 gene cluster in Streptomyces sp. FR-008 (Fig. 1a) (Chen et al., 2003). The deduced product of the 666 bp fscRI is a polypeptide of 222 aa with a molecular mass of 23.93 kDa. FscRI contains a PAS domain (aa 33–88) and an HTH domain of the LuxR type (aa 147–201). Amino acid comparisons indicated that FscRI is highly similar along its entire length to several characterized homologs of different polyene pathways, for example AmphRI (69 % identity), which regulates amphotericin (Caffrey et al., 2001), and PimM (69 % identity), which regulates pimaricin (Anton et al., 2007), and NysRI (66 % identity), an essential regulator of nystatin (Sekurova et al., 2004) (Fig. 1b). The high similarity between FscRI and these regulators of other polyenes suggests that FscRI may act as a positive regulator of FR-008 production.

In contrast, bioinformatics analysis indicated that FscRII, FscRIII and FscRIV are members of the LAL family. Each protein has an N-terminal AAA (ATPase associated with various cellular activities) domain (aa 2–173 for FscRII, 12–156 for FscRIII and 48–218 for FscRIV) and a C-terminal HTH domain of the LuxR type (aa 876–932 for FscRII, 951–1004 for FscRIII and 928–985 for FscRIV). Sequence comparisons revealed that FscRII, FscRIII and FscRIV are most similar to LAL-type proteins implicated in macrolide biosynthesis. FscRII and FscRIII are most similar, respectively, to NysRIII (51 % identity) and AmphRIII (48 % identity), and NysRI (44 % identity) and AmphRII (44 % identity). FscRIV is most similar to NysRI (56 % identity) and AmphRI (40 % identity). Interestingly, similarity was also found among these three regulators, with 31 % identity between FscRII and FscRIV, and 32 % identity between FscRII and FscRIV, implying that these three large regulatory proteins may share a common regulatory feature.

The four regulatory genes fscRIV–fscRI constitute a regulatory gene subcluster in the FR-008 locus with synteny to the regulatory gene subclusters in loci associated with biosynthesis of amphotericin (Carmody et al., 2004), nystatin (Brautaset et al., 2000) and NPP (Kim et al., 2009) (Fig. 1a). Although a complete sequence is not available for the candicidin and salinomycin loci (Campelo & Gil, 2002; Knirschova et al., 2007), available data indicate they have regulatory gene clusters with at least partial synteny to the other loci (Fig. 1a), suggesting that similar regulatory networks control synthesis of these polyene antibiotics.

Mapping the TSP of fscRI

We deduced from sequence analysis that fscRI may have an important role in the regulation of FR-008 biosynthesis. To better understand the transcription of fscRI, we mapped its TSP using 5'-RACE analysis with the gene-specific FscRI RACE Outer and Inner primers (Table S1), and localized it to a single guanine, 43 nt upstream of the putative start codon (Fig. 1c, d). Analysis of the sequence upstream of the TSP revealed a potential −10 box, GAAACG, and a potential −35 box, GCCGCA, separated by 17 nt from the −10 box (Fig. 1d), and these −10 and −35 sequences are partially similar to the consensus sequence for HrdB-recognized promoters.

Loss of FR-008 production in the ΔfscRI mutant

To determine the role of fscRI in the regulation of FR-008 biosynthesis, ΔfscRI was generated by deleting 243 bp of the fscRI coding sequence, corresponding to aa 123–203, which encompasses the HTH domain. The in-frame deletion was verified by Southern blot analysis and by PCR (Fig. S1). The ΔfscRI mutant strain demonstrated growth and morphological properties similar to those of the Streptomyces sp. FR-008 strain when grown on solid or in liquid medium (data not shown), suggesting that fscRI has no role in growth or differentiation.

Production of FR-008 by ΔfscRI was tested using Rhodotorula as the indicator strain. A large clear zone of growth inhibition was observed with the wild-type FR-008 strain, as expected (Fig. 2a). However, no growth inhibition of Rhodotorula was detected with ΔfscRI (Fig. 2b), suggesting
that deletion of \( fscRI \) abrogated FR-008 production. The failure of the \( fscRI \) mutant to synthesize FR-008 was supported by HPLC analysis. While three major peaks corresponding to the FR-008 complex were detected in the extracts of \( Streptomyces \) sp. FR-008 (Fig. 2a), no such peaks were detectable in the \( \Delta fscRI \) sample (Fig. 2b). These results indicated that \( fscRI \) is essential for the biosynthesis of the polyene antibiotic FR-008.
To confirm that deletion of the \textit{fscRI} coding sequence was directly responsible for the defect in FR-008 production, the chromosomal segment covering the coding sequence of \textit{fscRI} and its upstream region was cloned into pSET152, an integrative and conjugative plasmid (Kieser et al., 2000), and the resulting plasmid pRI-Com was introduced into \textit{ΔfscRI} to generate the complemented strain \textit{ΔfscRI-Com}. \textit{ΔfscRI-ComCK}, \textit{ΔfscRI} transformed with the vector pSET152 alone, was used as a control. Although \textit{ΔfscRI-Com} and \textit{ΔfscRI-ComCK} showed a wild-type phenotype for growth and morphological differentiation under the conditions tested (data not shown), the FR-008 complex was only restored in \textit{ΔfscRI-Com} (Fig. 2c), not in \textit{ΔfscRI-ComCK} (Fig. 2d), as indicated by both the bioassay and the HPLC analysis, confirming that FscRI is directly involved in the regulation of FR-008 biosynthesis.

**Fig. 2.** Analysis of FR-008 production (mAU) by HPLC and bioassay in the wild-type strain (a, e), \textit{ΔfscRI} (b), \textit{ΔfscRI-Com} (c), \textit{ΔfscRI-ComCK} (d), \textit{ΔfscRIV} (f), \textit{ΔfscRIV-Com} (g) and \textit{ΔfscRIV-ComCK} (h). The wild-type strain is shown at the top of each column to allow easier comparison of HPLC patterns. The wild-type strain FR-008 and its derivatives were grown on SFM agar medium for 4 days before extraction for HPLC analysis or before transplanting for the bioassay. The inset photographs (a–h) show growth inhibition of the indicator yeast \textit{Rhodotorula rubra} by strain FR-008 and its derivatives in the bioassay. Inhibition is indicated by the cleared zone.
Transcriptional analysis of structural genes in the FR-008 pathway in a ΔfscRI mutant

The FR-008 gene cluster includes six genes, fscA–fscF, encoding type I polyketide synthase subunits, two genes, fscTI and fscTII, that encode the putative ABC transport for the efflux of FR-008, and pabaB, which encodes an ADC synthase responsible for the biosynthesis of the starter unit PABA (Chen et al., 2003) (Fig. 3a). Deduced functions of additional genes in the cluster include attachment of mycosamine (fscMI), biosynthesis of mycosamine (fscMII and fscMIII), formation of a carboxyl group at C-18 (fscP), electron transfer in the P450 system (fscFE) and removal of aberrant intermediates (fscTE) (Chen et al., 2003). Upstream of fscMI are the four regulatory genes fscRI–fscRIV, with fscRI well separated from the other three genes by a 558 bp intergenic region. Also, fscO and pabC, the two genes situated upstream of fscRI, encode an FDA-dependent monooxygenase and an ADC lyase, respectively (Chen et al., 2003; Zhang et al., 2009).

Because of the loss of FR-008 production in the ΔfscRI mutant and the high similarity of FscRI to PimM, which regulates genes of the pimaricin pathway (Antón et al., 2007), we hypothesized that at least some genes of the FR-008 pathway would be regulated by FscRI. To identify these genes, total RNA was extracted from ΔfscRI and from control strains that produced FR-008, and gene-specific primers for RT-PCR and real-time PCR analysis were designed (Table S1). Expression of hrdB, encoding the housekeeping sigma factor of Streptomyces, was used as an internal control for both analyses.

The transcription of structural genes was first analysed by RT-PCR (Fig. 3b). The transcription of fscO and pabC in ΔfscRI was comparable to that in the wild-type Streptomyces sp. FR-008, as judged by the bands of similar intensity. In contrast, the transcription of all other structural genes was either undetectable or decreased significantly in ΔfscRI, compared with the parental strain, suggesting that FscRI directly or indirectly controls the 15 genes from fscMI to fscMIII. Transcription of these genes was partially to fully restored in the complemented strain ΔfscRI-Com (Fig. 3b).

To investigate more closely the relative changes in gene expression levels caused by deletion of fscRI, we performed real-time PCR analysis (Fig. 3c). To facilitate comparison, the level of expression for each gene in ΔfscRI was arbitrarily set to 1. The mean (±SD) level of expression of fscO and pabC in strain FR-008 was, respectively, 1.53 ± 0.8 and 0.66 ± 0.1 times the level in ΔfscRI, indicating that FscRI has little or no regulatory effect on these two genes, consistent with the RT-PCR analysis. In sharp contrast, dramatically higher expression levels were detected for the five consecutive genes fscMI–fscTE, ranging from 379.9 ± 30.9 (fscP) to 169.48 ± 47.4 (fscFE) in the wild-type strain. High levels were also measured for fscA (178.4 ± 30.1), fscTI (207.0 ± 31.4), fscD (189.14 ± 13.1), fscE (175.16 ± 9.6), fscB (119.77 ± 12.8), fscC (175.93 ± 48.1) and fscMIII (106.82 ± 13.1) in strain FR-008. Although the difference in expression of pabaB was lower than for the majority of the genes, it was still approximately 34 times higher in strain FR-008 than in ΔfscRI (Fig. 3c). The levels of expression of these genes in the complemented strain ΔfscRI-Com were partially restored (Fig. 3c).

Based on their genetic organization, short intergenic sequences and similar levels of transcription, genes from fscMI to fscTE may be co-transcribed. To test this hypothesis, we carried out additional RT-PCR studies using primers that would detect transcription between adjacent genes. Amplicons of the expected sizes were obtained between fscMI and fscMII, fscMII and fscP, fscP and fscFE, and fscFE and fscTE (Fig. S4), suggesting these genes form an operon.

FscRI regulates fscRIV

The above data indicate that FscRI regulates the expression of 15 consecutive structural genes in the FR-008 pathway, so transcription of the four regulatory genes was similarly evaluated. Using primers to an undeleted portion of fscRI, the intensity of the amplicon for fscRI in ΔfscRI was similar to that of the wild-type strain (Fig. 3b), suggesting that fscRI is not autoregulatory. Transcription of fscRII and fscRIV was also similar in both strains. However, transcription of fscRIV was nearly undetectable in the ΔfscRI mutant compared with the wild-type FR-008 strain (Fig. 3b). By real-time PCR, the minimal differences in expression of fscRI (0.58 ± 0.1), fscRII (1.46 ± 0.1) and fscRIV (1.36 ± 0.2) in FR-008 compared with ΔfscRI were consistent with the RT-PCR analysis (Fig. 3d). In contrast, a decrease of 7.8-fold for fscRIV was present in the mutant strain, and the reduced expression in ΔfscRI was partially restored in the complemented strain (Fig. 3d), confirming that FscRI regulates fscRIV, but not fscRII, fscRIV or fscRI itself.

Reduced production of FR-008 in the ΔfscRIV mutant

The finding that fscRIV is regulated by FscRI prompted us to investigate whether fscRIV also has a role in FR-008 production. The ΔfscRIV mutant was generated by deleting 2559 bp of its coding sequence, corresponding to aa 109–961, including most of the AAA domain and more than half of the HTH domain. The in-frame deletion of the fscRIV sequence was verified by PCR analysis (Fig. S2).

Production of FR-008 by ΔfscRIV was tested using Rhodotorula as the indicator strain. Compared with the large zone generated by the wild-type strain (Fig. 2e), an inhibition zone of reduced size was detected with ΔfscRIV (Fig. 2f), suggesting that ΔfscRIV had reduced FR-008 production. The decrease in FR-008 production by the fscRIV mutant was confirmed by HPLC analysis, with only small peaks corresponding to the FR-008 complex detected in the ΔfscRIV sample (Fig. 2f), in contrast to the major peaks detected in extracts from Streptomyces sp. FR-008 (Fig. 2e). These results indicated that fscRIV is required to produce normal levels of FR-008. To confirm that deletion of the
The fscRIV sequence was responsible for the reduced production of FR-008, the complemented strain ΔfscRIV-Com was generated. As indicated by bioassay and HPLC analysis, FR-008 production was restored in ΔfscRIV-Com (Fig. 2g) to a level comparable to that in wild-type FR-008, but it was not restored in ΔfscRIV-ComCK (Fig. 2h), which is ΔfscRIV transformed with pSET152 only, confirming that FscRIV is directly involved in the regulation of FR-008 biosynthesis.
Transcriptional analysis in the ΔfscRIV mutant

Next, we examined the effects of deletion of fscRIV on expression of individual genes in the FR-008 pathway. RT-PCR analysis (Fig. 4a) showed that transcription of fscO was essentially unchanged between ΔfscRIV and the wild-type strain, similar to the findings for this gene with ΔfscRI. However, in contrast to ΔfscRI, transcription of pabC was greatly diminished in ΔfscRIV, implying that FscRIV regulates pabC. As in ΔfscRI, the amplicons of all other structural genes from fscMI to fscMIII were either undetectable or decreased significantly in the ΔfscRIV mutant compared with those in the wild-type FR-008. The transcription of genes that were downregulated in the ΔfscRIV mutant was restored in the complemented strain, ΔfscRIV-Com, to a level comparable to that in the wild-type strain, suggesting that FscRIV, like FscRI, also controls genes fscMI–fscMIII.

Real-time PCR was also performed to determine relative expression levels in the FR-008 gene cluster, with the expression level for each gene in ΔfscRIV arbitrarily set to 1 (Fig. 4b). Expression of fscO was nearly unchanged between strain FR-008 and ΔfscRIV, consistent with the RT-PCR analysis. Expression of pabC was 15.02 ± 1.8-fold greater in the wild-type strain than in ΔfscRIV, in agreement with RT-PCR analysis. Although a similarly high level of expression was obtained for fscMI (18.15 ± 4.27), fscMII (16.86 ± 6.63), fscP (17.34 ± 5.41), fscFE (16.05 ± 4.35) and fscTE (15.98 ± 4.65) in strain FR-008, these values were much lower than the fold differences in expression between strain FR-008 and ΔfscRI, which ranged from approximately 169 to 380 for the same genes (Fig. 3c). Additionally, while the expression level of pabAB was only 34-fold higher in Streptomyces sp. FR-008 relative to ΔfscRI (Fig. 3c), this difference was still greater than the difference relative to ΔfscRIV (15.22 ± 4.19) (Fig. 4b). The fscA gene was the most highly upregulated (40.59 ± 8.43) gene in the cluster in the FR-008 strain, when compared with ΔfscRIV, followed by roughly similar differences for fscTI (10.4 ± 0.1), fscTII (15.86 ± 3.8), fscC (28.28 ± 9.2), fscB

![Fig. 4. Gene expression patterns in the ΔfscRIV mutant. (a) RT-PCR analysis of gene transcription. For each gene, lanes 1, 2 and 3 indicate the wild-type strain FR-008, ΔfscRIV and ΔfscRIV-Com, respectively. (b, c) Real-time PCR analyses. For each gene, the expression level in the ΔfscRIV deletion mutant was arbitrarily set to 1. The y-axis shows the fold change in expression levels in FR-008 (white bars) and ΔfscRIV-Com (light grey bars) over levels in ΔfscRIV (black bars). (b) Expression of structural genes in the FR-008 pathway. (c) Expression of regulatory genes in the FR-008 pathway.](http://mic.sgmjournals.org)
(24.35 ± 11.2), fscF (12.42 ± 0.1), fscE (17.79 ± 4.7), fscD (25.18 ± 7.1) and fscMII (19.23 ± 6.1) (Fig. 4b), in agreement with RT-PCR analysis.

Altogether, these data confirm that FscRIV regulates pabC and genes fscMI–fscMII. However, comparison of the results obtained with the ΔfscRI and ΔfscRIV mutant strains reveals that deleting fscRI had a dramatically greater impact on gene expression levels than did deletion of fscRIV, with 10- to 20-fold larger reductions in the expression of genes fscTII, fscTI and fscMI–fscTE in ΔfscRI, suggesting that FscRI has a much stronger regulatory effect than FscRIV on gene expression in the FR-008 pathway.

FscRIV regulates fscRI

The finding that fscRIV was downregulated in ΔfscRI prompted us to test whether any regulatory genes were downregulated in the ΔfscRIV mutant. The transcription of fscRI–fscRIVIII was evaluated first by RT-PCR (Fig. 4a). The amplicon for fscRI in ΔfscRIV was lower in intensity than that in the wild-type strain, while similar levels of transcription were observed for both fscRII and fscRIII in the wild-type FR-008 and ΔfscRIV strains (Fig. 4a), suggesting that FscRIV regulates fscRI, but not fscRII or fscRIII. To determine the relative expression level of fscRI–fscRIII, their transcription was quantified by real-time PCR (Fig. 4c). While only a less than twofold difference of expression was measured for fscRII and fscRIII in strain FR-008 compared with ΔfscRIV, the expression level of fscRI was 11.87 ± 3.7-fold greater in the wild-type FR-008 strain, and the level was restored in the complemented strain ΔfscRIV-Com, confirming that FscRIV regulates fscRI, but not fscRII or fscRIII. Because there is only a short coding sequence remaining in ΔfscRIV, we were unable to detect the expression of fscRIV itself in the ΔfscRIV mutant, and therefore we do not know whether fscRIV is autoregulatory. However, overall, these data indicate that fscRI and fscRIV are interregulatory, and that expression of fscRII and fscRIII is independent of fscRI and fscRIV.

Expression of fscRI restores production of the antibiotic FR-008/candicidin in the ΔfscRIV mutant

As fscRI and fscRIV appear to be mutually regulated, we next investigated whether these two genes can be cross-complemented. pRI-Com, which is able to restore FR-008/candidin production in the ΔfscRI mutant, was introduced into the ΔfscRIV mutant to obtain ΔfscRIV-Com-II. Similarly, pRIV-Com, which can complement the ΔfscRIV phenotype, was introduced into the ΔfscRIV mutant to obtain ΔfscRIV-Com-II. Production of FR-008 by ΔfscRI-Com-II and ΔfscRIV-Com-II was tested by bioassay using Rhodotorula as the indicator strain (Fig. S5). Compared with the clear zone generated by the wild-type and ΔfscRI-Com samples, no inhibition zone was detected with ΔfscRI-Com-II (Fig. S5), suggesting that fscRIV could not complement fscRI under these conditions. In contrast, an inhibition zone, obviously larger than that produced by ΔfscRIV and comparable to that of the wild-type, was detected in ΔfscRIV-Com-II, suggesting that fscRI is able to complement fscRIV. These results indicate that the decreased expression of fscRI in ΔfscRIV accounts largely for the reduced production of FR-008 antibiotic by this mutant, but that the abrogated production of FR-008 by ΔfscRI is not due to the reduced expression of fscRIV.

FscRI binding to the regions upstream of fscRIV and other genes

The above data indicate that FscRI activates the transcription of a consecutive set of 16 genes from fscRIV to fscMIII, so we speculated that FscRI may interact directly with one or more of the promoters in this locus. To investigate the targets of FscRI, we amplified DNA segments covering the upstream regions for 13 genes to use as probes in EMSAs, excluding genes that had only a very short (fscRIII, fscMII and fscTE) or no intergenic region (fscRII, fscE and fscF), or whose upstream sequence is not available (fscMIII). When incubated with purified FscRI, obvious shifting was observed with probes containing the intergenic regions for pabC, fscRIV (fscMI), pabAB, fscA, fscB and fscD (Fig. 5), indicating that FscRI interacts directly with them. Competition tests were performed to determine whether the binding of FscRI to these probes is specific. With the addition of the same unlabelled probe, the intensity of the shifted band decreased gradually, suggesting that FscRI binds specifically to the promoters of fscRIV, pabC and pabAB (Fig. 6). The binding of FscRI to its own promoter was also tested, but no binding was detected (Fig. 5). Given that FscRI binds the intergenic sequences between several other genes, this finding suggests that FscRI is not autoregulatory, consistent with the transcriptional data.

Because of the high sequence similarity between FscRI and PimM, and the availability of the consensus sequence for PimM binding (Santos-Aberturas et al., 2011a, b), we searched for sequences similar to the PimM binding consensus in the regions upstream of pabC, fscRIV (fscMI), pabAB, fscA, fscB and fscD, regions that were bound by FscRI (Fig. 5). A single potential FscRI binding site was detected in the sequence upstream of fscA, fscD and fscRIV (fscMI) (Fig. 7a), while two such sites were identified upstream of fscB, suggesting that these sites mediate the regulatory effect of FscRI. Surprisingly, no sequence similar to the PimM consensus was mapped in the upstream region of pabC or pabAB, although FscRI binds these regions specifically (Fig. 6). To verify that the sequences similar to the PimM consensus are authentic FscRI binding sites, EMSAs were carried out using oligonucleotides containing point mutations. Mutating bases in the middle of the predicted FscRI sites upstream of fscRIV and fscA inhibited FscRI binding (Fig. 7b–d), confirming that these sequences are valid FscRI binding sites.
We also expanded the search to the whole FR-008 cluster, and six additional sites similar to the PimM consensus recognition site were revealed (Fig. 7a). One potential FscRI site was mapped in the upstream region of fscO. However, the probe containing this site was only faintly shifted by FscRI (Fig. 5), possibly due to fewer matches with the consensus. One predicted FscRI site was found in the region far upstream of fscRI, located between bases −551 and −536 (relative to the start codon). Although the probe used for EMSA (Fig. 5) did not contain this sequence and we cannot exclude the possibility that FscRI binds here, we anticipate that this site does not participate in the regulation of fscRI, as we found that fscRI is not autoregulatory, at least under the conditions tested. There is one predicted FscRI site in the coding sequence of fscRIV, and interestingly, this site overlaps the stop codon, and thus may serve as an additional control for expression of fscRIV. One such site was also identified in the coding sequence of fscF, and two in the coding sequence of fscE (Fig. 7a).

To determine the location of the FscRI site relative to the TSPs of fscRIV and fscMI in the divergent region, the TSP for both genes was determined using 5′-RACE analysis. A cytosine, 9 nt downstream of the predicted start codon, was mapped as the TSP for fscRIV, while the TSP for fscMI was localized to a guanine (in the complementary strand), 8 nt upstream of the start codon (Fig. S6). The FscRI site is therefore located 51 and 139 nt, respectively, upstream of the TSPs of fscRIV and fscMI.

**DISCUSSION**

The FR-008 pathway contains four regulatory genes encoding proteins from two different families of regulators. FscRI is highly similar to several transcriptional regulators with an N-terminal PAS and a C-terminal HTH domain that regulate different macrolide pathways, while FscRII, FscIII and FscIV are large proteins belonging to the LAL family. Our studies indicate that FscRI is essential for the biosynthesis of FR-008, with disruption of fscRI abrogating FR-008 production in the ΔfscRI mutant. However, deletion of fscRIV only reduced FR-008 biosynthesis, suggesting that FscRI and FscRIV have differential regulatory effects on the
FR-008 pathway. This hypothesis was supported by our transcription data, which demonstrated that, although both FscRI and FscRIV regulate the 15 consecutive structural genes from \( fscMI \) to \( fscMIII \), their impact on expression levels differs by about 10-fold (Figs 3c and 4b), with \( fscMI – fscMIII \) being more highly regulated by FscRI than by FscRIV. Interestingly, our data also suggested that expression of \( fscRI \) and \( fscRIV \) is interregulated, while transcription of \( fscRII \) and \( fscRIII \) is independent of FscRI and FscRIV.

Based on the finding that FscRI and FscRIV are mutually regulated, and that FscRI and FscRIV exhibit different levels of regulation on the structural genes of the FR-008 pathway, we hypothesized that FscRI may mediate the regulatory effect of FscRIV. In support of this, we found that the reduced expression of \( fscRI \) in \( \Delta fscRIV \) is associated with decreased expression of FscRI target genes. About a 10-fold reduction in expression was measured for \( fscRI \) in the \( \Delta fscRIV \) mutant, comparable to the level of reduction in the expression of most structural genes in \( \Delta fscRIV \). This hypothesis is reinforced by the finding that expression of \( fscRI \) in \( \Delta fscRIV \) could restore the production of antibiotic FR-008/candicidin to a level comparable to that of the wild-type strain (Fig. S5). Additionally, an approximate sevenfold decrease was observed for the expression of \( fscRIV \) in the \( \Delta fscRI \) mutant, and this decrease may have further reduced expression of \( fscRI \), and consequently the expression of FscRI target genes. Because FscRI regulates FscRIV, it is consistent that antibiotic production was not reduced in the \( \Delta fscRI \) mutant.
restored when ΔfscRI was transformed with pRIV-Com, in which fscRIV expression is driven from its native promoter. It is possible that forced expression of fscRIV from a promoter not regulated by FscRI could complement ΔfscRI. Although fscRIV, fscRIII and fscRII appear to form an operon, deletion of fscRI had no detectable effect on fscRIII or fscRII.

Our EMSAs showed that FscRI interacts directly with the intergenic regions upstream of pabC, fscRIV/fscMI, pabAB, fscA, fscB and fscD, implying that these regions contain sequences required for FscRI binding. By searching for sequences similar to the PimM consensus, putative FscRI sites were identified in the regions upstream of fscA, fscB (fscBI/2), fscD and fscO, and in the divergent promoter region between fscRIV and fscMI (Fig. 7a), in agreement with a previous report (Santos-Aberturas et al., 2011a). Although FscRI interacts directly with the upstream sequences of seven genes, not every DNA fragment bound by FscRI possesses a detectable PimM site, which was the case for pabC and pabAB, whose upstream sequences were bound specifically by FscRI (Fig. 6). We could not find sequences similar to the PimM consensus, even using reduced stringency, upstream of pabC and pabAB, suggesting that FscRI can bind more divergent sequences. Our transcriptional analysis indicates that FscRI controls the expression of 16 genes in the FR-008 cluster. However, as FscRI interacts only with the upstream sequences of a few genes, other genes in the pathway may be co-transcribed with those genes that are directly regulated by FscRI. From our analyses (Fig. S4), this appears to be true for fscMI–fscTE, and our preliminary data also suggest that fscD–fscF, and fscB and fscC may form operons.

Although most genes in the FR-008 pathway are regulated by both FscRI and FscRIV, albeit to different levels, the direct targets of FscRIV remain obscure. The only two genes that demonstrated markedly different expression patterns in ΔfscRI and ΔfscRIV were fscRI and pabC. Although there is an FscRI binding site in the pabC promoter, it is more likely that pabC is a greater regulatory target of FscRIV than FscRI, based on comparison of its expression levels in ΔfscRI and ΔfscRIV. We hypothesize that fscRI and pabC are direct regulatory targets of FscRIV, although this hypothesis needs to be confirmed by EMSA. We are currently working on expressing the HTH domain of fscRIV to determine whether FscRIV binds the promoters of fscRI and pabC, and other genes in the FR-008 pathway.

The four regulatory genes fscRI–fscRIV of the FR-008 pathway are highly similar to the four regulatory genes nysRI–nysIV of the nystatin pathway (Fig. 1a), whose roles in the biosynthesis of nystatin were investigated in vivo (Sekurova et al., 2004), and all of which were found to be necessary for efficient nystatin production. Mutation of nysIV, which is homologous to fscRII, resulted in production of nystatin levels that were only 2% of those in the parental strain (Sekurova et al., 2004), and this is similar to our findings that production of FR-008 was not detectable by bioassay or by HPLC analysis in ΔfscRI (Fig. 2b). Interestingly, a nysRI mutant produced nystatin levels that were only 0.5% of normal (Sekurova et al., 2004), whereas deletion of the homologous gene, fscRIV, led to reduced, but still substantial, levels of FR-008 production. Although we did not quantify the amount of FR-008 produced by ΔfscRIV, it appears that ΔfscRIV produces FR-008 at a level approximately 10% that of the wild-type, as judged from our HPLC analysis (Fig. 2f), implying that the role of FscRIV in the FR-008 pathway differs from that of NysRI in the nystatin pathway.

The intergenic region of nysDI–nysA contains divergent promoters, and a sequence similar to the PimM consensus was identified in this region (Santos-Aberturas et al., 2011a), which may be a potential NysRIV binding site owing to the high similarity of this regulator to PimM. Using xyIE as a reporter, fusion plasmids were constructed to test the expression of selected genes in mutant strains of nysRI–nysIV (Sekurova et al., 2004). Surprisingly, nysDI seems to have a slightly enhanced expression in both a nysRI and a nysRIV mutant strain (Sekurova et al., 2004), whereas expression of nysA was greatly decreased (Sekurova et al., 2004), indicating that NysRI and NysRIV activate nysA but repress nysDI. Another PimM-like site was identified between nysH and nysDIII which contains divergent promoters (Santos-Aberturas et al., 2011a). However, NysRIV (corresponding to FscRI) appears to regulate nysH only (Sekurova et al., 2004), whereas NysRI (corresponding to FscRIV) affected transcription of both nysH and nysDIII, but only slightly (Sekurova et al., 2004). Due to the limited number of genes tested (Sekurova et al., 2004), the regulatory effect of NysRI and NysRIV on other genes in the Nys cluster is unknown. However, from the results of our transcriptional analysis, it seems that there are considerable differences between regulation of the nystatin and FR-008/candicidin systems. We observed a generally positive regulatory role of FscRI and FscRIV for almost all structural genes, including those that have a divergent promoter in the FR-008 pathway. However, some of the differences may also be due to the sensitivity of the methods used, as we believe that real-time PCR is more sensitive than reporter assays.

Overall, we made substantial inroads into understanding gene expression patterns in the FR-008/candicidin pathway and with regard to the regulatory roles of fscRI and fscRIV. We found that the 15 genes, fscMII–fscMIII, are regulated by both FscRI and FscRIV, but to different levels, and most interestingly we determined that fscRI and fscRIV are mutually regulated. The fsc cluster represents an attractive system for the study of gene regulation in a complex antibiotic pathway.

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