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GENETICS AND MOLECULAR BIOLOGY

A Hierarchical Network of Four Regulatory Genes Controlling Production of the Polyene Antibiotic Candicidin in *Streptomyces* sp. Strain FR-008

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ABSTRACT The four regulatory genes *fscR1* to *fscR4* in *Streptomyces* sp. strain FR-008 form a genetic arrangement that is widely distributed in macrolide-producing bacteria. Our previous work has demonstrated that *fscR1* and *fscR4* are critical for production of the polyene antibiotic candicidin. In this study, we further characterized the roles of the other two regulatory genes, *fscR2* and *fscR3*, focusing on the relationship between these four regulatory genes. Disruption of a single or multiple regulatory genes did not affect bacterial growth, but transcription of genes in the candicidin biosynthetic gene cluster decreased, and candicidin production was abolished, indicating a critical role for each of the four regulatory genes, including *fscR2* and *fscR3*, in candicidin biosynthesis. We found that *fscR1* to *fscR4*, although differentially expressed throughout the growth phase, displayed similar temporal expression patterns, with an abrupt increase in the early exponential phase, coincident with initial detection of antibiotic production in the same phase. Our data suggest that the four regulatory genes *fscR1* to *fscR4* have various degrees of control over structural genes in the biosynthetic cluster under the conditions examined. Extensive transcriptional analysis indicated that complex regulation exists between these four regulatory genes, forming a regulatory network, with *fscR1* and *fscR4* functioning at a lower level. Comprehensive cross-complementation analysis indicates that functional complementation is restricted among the four regulators and unidirectional, with *fscR1* complementing the loss of *fscR3* or *-4* and *fscR4* complementing loss of *fscR2*. Our study provides more insights into the roles of, and the regulatory network formed by, these four regulatory genes controlling production of an important pharmaceutical compound.

IMPORTANCE The regulation of antibiotic biosynthesis by *Streptomyces* species is complex, especially for biosynthetic gene clusters with multiple regulatory genes. The biosynthetic gene cluster for the polyene antibiotic candicidin contains four consecutive regulatory genes, which encode regulatory proteins from different families and which form a subcluster within the larger biosynthetic gene cluster in *Streptomyces* sp. FR-008. Syntenic arrangements of these regulatory genes are widely distributed in polyene gene clusters, such as the amphotericin and nystatin gene clusters, suggesting a conserved regulatory mechanism controlling production of these clinically important medicines. However, the relationships between these multiple regulatory genes are unknown. In this study, we determined that each of these four regulatory genes is critical for candicidin production. Additionally, using transcriptional analyses, bioassays, high-performance liquid chromatography (HPLC) anal-

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ysis, and genetic cross-complementation, we showed that FscR1 to FscR4 comprise a hierarchical regulatory network that controls candicidin production and is likely representative of how expression of other polyene biosynthetic gene clusters is controlled.

KEYWORDS *Streptomyces*, regulation, antibiotic, polyene, regulation of gene expression

The actinomycete bacteria, especially the soil-dwelling *Streptomyces*, are a large family of microbes capable of producing compounds with clinical application, including anti-infectious agents (1). The agents with antifungal activity are mostly polyene antibiotics, which comprise a family of type I polyketide macrolide ring compounds with backbones of 20 to 40 or more carbons containing several conjugated double bonds (2). Polyene antibiotics can bind specifically to ergosterol and alter the cellular functions in which ergosterol is involved (3–6).

In *Streptomyces* species, genes responsible for the biosynthesis of an antibiotic are usually clustered together, forming a biosynthetic gene cluster (BGC). However, production of antibiotics can be regulated at different levels, including through regulatory genes within the cluster and beyond, forming an apparent regulatory cascade (7). In addition to structural genes that are devoted to the assembly or formation of the main chemical structure, a specific BGC will often contain the regulatory genes that constitute the lower levels of the regulatory cascade controlling production of the metabolite, such as *actII-ORF4* for actinorhodin and *redD* for undecylprodigiosin in *Streptomyces coelicolor*, as well as other regulators (7–10). However, regulatory factors outside a specific BGC, for example, pleiotropic or global regulatory factors such as signal transduction systems and sigma factors, can also affect the biosynthesis of a specific compound (7, 11–14), and in some circumstances, the pathway-specific regulatory factors mediate the regulatory effects of these pleiotropic or global regulatory factors (15–17).

The best-known polyene antibiotics include amphotericin, candicidin, nystatin, and pimaricin (2, 18–20). Gene clusters for the biosynthesis of these polyene antibiotics have been sequenced (21–28), and multiple regulatory genes were identified within these clusters. The compound FR-008 is a heptaene macrolide antibiotic produced by *Streptomyces* sp. strain FR-008 and is identical to candicidin in structure (26, 28). The genome of *Streptomyces* sp. FR-008 has been sequenced (GenBank accession numbers CP009802 to CP009804) (28), and in addition to the linear chromosome, the FR-008 genome harbors two linear plasmids, which altogether comprise a total genome length of 7.2 Mb; this is one of the smallest *Streptomyces* genomes thus far reported (28, 29). Bioinformatic analysis revealed that the *Streptomyces* sp. FR-008 genome carries 23 putative secondary metabolic gene clusters, only two of which were annotated as encoding type I polyketide synthases (PKS), including the one for the biosynthesis of candicidin. *Streptomyces* sp. FR-008 also carries a cluster for the biosynthesis of another antifungal agent, antimycin (26, 28, 30). In the putative BGC for candicidin, which extends more than 100 kb in length and encompasses 21 genes (see Fig. S1 in the supplemental material), four consecutive regulatory genes, *fscR1* to *fscR4*, were found to form a regulatory subcluster (26, 28) encoding transcriptional regulators from two different families in the FR-008 pathway. FscR1 is a relatively small protein of 231 amino acids (aa) containing a PAS domain and a helix-turn-helix (HTH) domain of the LuxR type, for which the archetype is PimM (31, 32); FscR2, FscR3, and FscR4 are relatively large proteins of more than 900 aa and are members of the large ATP-binding family of regulators of the LuxR type (LAL) (33), with each containing an AAA-ATPase domain and an HTH domain of the LuxR type and FscR3 additionally containing a DNA polymerase III domain (see Fig. S2 in the supplemental material). Orthologous genes (see Fig. S3 and S4 in the supplemental material) (34) form syntenic clusters (see Fig. S5 in the supplemental material) in the pathways for multiple polyene antibiotics, such as amphotericin (24, 35), candicidin (25, 27), nystatin (23, 36), and salinomycin (37, 38),

implying that the strategy for controlling expression of polyene antibiotics is likely conserved. The roles of the four orthologous regulatory genes in the nystatin biosynthetic cluster have been investigated, and it was found that the four *nysR* genes are involved in production of nystatin (36, 39, 40). Our previous study showed that at least two of the four regulatory genes in the FR-008 BGC, *fscR1* and *fscR4*, are required for the biosynthesis of candicidin, and interestingly, these two regulatory genes are also cross-regulated (34). However, the relationship among, and the regulatory network formed by, these four regulatory genes had not been characterized. In this study, we further characterized the roles of the remaining two regulatory genes, *fscR2* and *fscR3*, and investigated the relationship among these four regulatory genes in the polyene FR-008 BGC. Our study revealed an intraregulatory network formed by multiple regulatory genes of the same pathway that control polyene production in *Streptomyces*.

RESULTS

The four regulatory genes *fscR1* to *fscR4* have similar temporal transcription patterns. As secondary metabolites, antibiotics are usually produced by *Streptomyces* species after the organisms enter stationary phase on solid medium, coincident with morphological differentiation (41, 42). To investigate the temporal expression of these regulatory genes and its association with production of candicidin, we first prepared the growth curve of the wild-type strain of *Streptomyces* sp. FR-008 in liquid YEME, a rich growth medium (43). As shown in Fig. 1A (left panel), the wild-type strain displayed a growth curve typical of *Streptomyces* (43, 44). Production of candicidin was not yet detectable in the 24-h sample but was detectable for samples taken at 36 h, as reflected by a clear, although small, zone of inhibition of the indicator yeast strain (Fig. 1B). Enhanced levels of candicidin were detected in samples taken at 42 h and thereafter, as judged by the larger zones of inhibition (Fig. 1B), indicating that candicidin is produced during the exponential phase of growth.

Streptomyces sp. FR-008 harbors an additional BGC that produces another antifungal agent, antimycin (30), the production of which is also regulated by FscR1 (45). To investigate whether antimycin contributed to the inhibition of the indicator strain (*Rhodotorula rubra*) in the bioassay, we tested the sensitivity of the indicator strain to antimycin at different concentrations (see Fig. S6 in the supplemental material). Our analysis showed that whereas the wild-type FR-008 strain generated a clear zone of inhibition, antimycin did not produce a detectable zone of inhibition at concentrations up to 10 μg/ml, indicating that under the conditions tested, the indicator strain is not sensitive to antimycin. These results indicate that the bioassay data with *Streptomyces* sp. FR-008 reflect the level of candicidin.

To examine the temporal expression of the regulatory genes during growth, total RNA was prepared using samples collected at different time points as shown in Fig. 1A, and real-time PCR was performed. To facilitate the analysis, the quantitative PCR (qPCR) value of each gene was first normalized by comparison with *hrdB* transcription, and then the expression level of each gene at 24 h was arbitrarily set to 1 (Fig. 1C). Similar temporal expression patterns were observed for these four regulatory genes, with maximum expression detected at 36 h and decreasing expression afterwards (Fig. 1C); this pattern was consistent with the detectable production of candicidin at this time point (Fig. 1B), strongly suggesting a role for these regulatory factors in the biosynthesis of candicidin.

The four regulatory genes *fscR1* to *fscR4* are differentially expressed. Our above data indicated similar temporal expression patterns for the four regulatory genes, with each exhibiting induction of greater than 100-fold by 36 h. However, as the fold changes shown in Fig. 1C were determined independently for each gene, i.e., relative to the expression level of that gene at 24 h, these results did not allow for direct comparison of the relative expression levels between genes. To compare their relative levels, the expression level of *fscR1* at each time point was arbitrarily set to 100 and was used as the control to evaluate the expression of the other three regulatory genes, *fscR2* to *fscR4*, at the same time point (Fig. 1D; see Fig. S7 in the supplemental material).

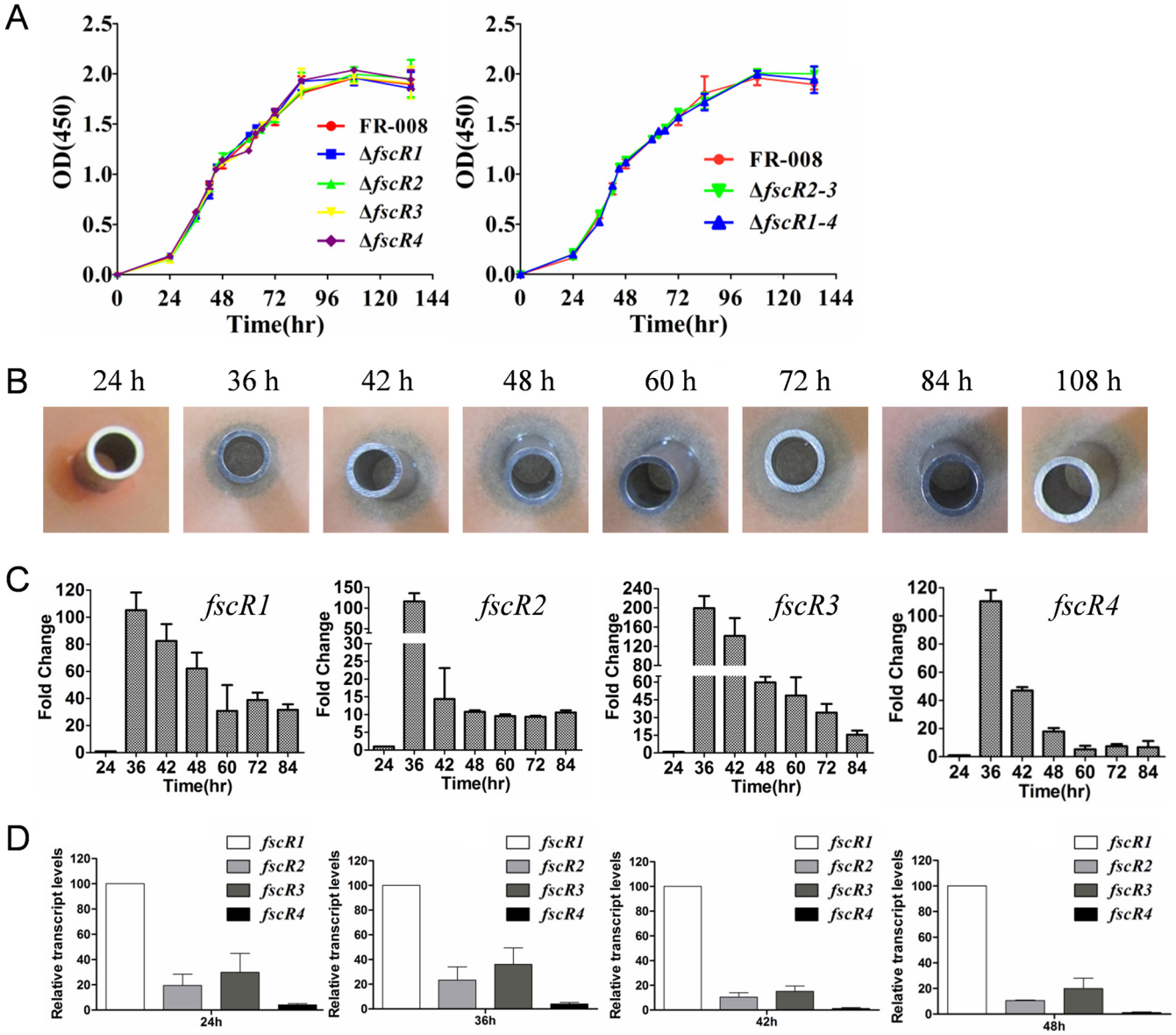

FIG 1 Growth phenotypes of mutants and temporal expression patterns of regulatory genes of the FR-008 pathway. (A) Growth curves of *Streptomyces* strains in YEME liquid medium. Left panel, wild-type *Streptomyces* sp. strain FR-008 and single mutant strains with deletion of one regulatory gene; right panel, FR-008 and mutant strains with deletion of more than one regulatory gene. (B) Bioassay of samples of the wild-type strain taken at the indicated time points during growth. (C) Temporal expression pattern of the four regulatory genes *fscR1* to *fscR4* in *Streptomyces* sp. FR-008 cultured in YEME liquid medium. RNA samples were isolated at the indicated times, and expression of *hrdB*, which encodes the major sigma factor, was used as an internal control. The expression level of each gene at 24 h was arbitrarily set to one. The *y* axis shows the fold change in expression at the indicated time over the level at 24 h. Results are the means (± standard deviations [SD]) from triplicate biological experiments. (D) Comparison of the relative expression of *fscR1* to *fscR4* in the wild-type strain during growth in YEME liquid medium. In this assay, expression of *hrdB*, which encodes the major sigma factor, was used as an internal control, and the expression level of *fscR1* at each time point was arbitrarily set to 100. The *y* axis shows the fold change in expression of each gene over the expression of *fscR1* at the indicated times. Results are the means (±SD) from triplicate biological experiments.

Collectively, our analysis indicated that the four regulatory genes of FR-008 pathway are differentially expressed, with *fscR1* expressed at the highest level, followed in order by *fscR3*, *fscR2*, and *fscR4*, suggesting potentially different roles for these regulatory genes in controlling candicidin biosynthesis.

Temporal expression patterns of structural genes in the FR-008 pathway. In addition to the four regulatory genes *fscR1* to *fscR4*, the FR-008 BGC encompasses another 17 genes involved in the construction, modification, and efflux of the chemical compounds: *fscA* to *fscF* (46), encoding the type I PKS subunits; *fscMI*, *fscMII*, and *fscMIII* for biosynthesis and attachment of mycosamine (47); *fscP* for formation of a carboxyl

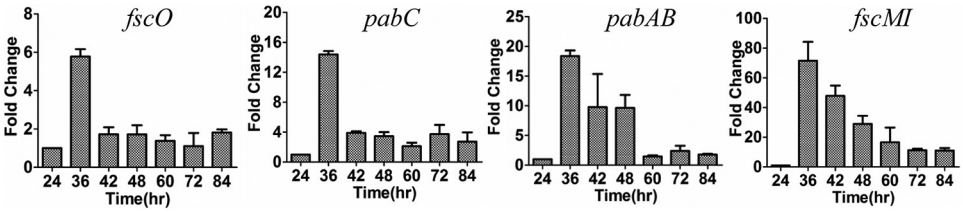

FIG 2 Temporal expression pattern of structural genes of the FR-008 pathway in the wild-type *Streptomyces* sp. strain FR-008 cultured in YEME liquid medium. RNA samples were isolated at the indicated times. Expression of *hrdB*, which encodes the major sigma factor, was used as an internal control. The expression level of each gene at 24 h was arbitrarily set to one. The *y* axis shows the fold change in expression level at the indicated times over the level at 24 h. Results are the means (±SD) from triplicate biological experiments.

group (48); *fscFE* for electron transfer; *fscTE* for removal of aberrant intermediates (49); *pabAB* (47), encoding an 4-amino-4-deoxychorismate (ADC) synthetase; *fscTI* and *fscTII*, encoding a putative ABC transporter (50); and *fscO* and *pabC*, encoding a flavin adenine dinucleotide (FAD)-dependent monooxygenase and an ADC lyase, respectively (26, 28, 51). To investigate the temporal expression pattern of these structural genes, real-time PCR was performed using the same sets of RNAs used for analyzing the regulatory genes, with the level of each gene at 24 h arbitrarily set to one. Our data indicated that all structural genes were obviously induced at 36 h, in a temporal expression pattern similar to that of the regulatory genes and also consistent with the detectable level of candicidin at 36 h. However, based on differences in their induction levels, the structural genes were categorized into three groups. Genes of the first group were only slightly induced at 36 h, as represented by *fscO* (Fig. 2), the expression of which increased to 5.7 ± 0.4 at 36 h and was then reduced to near-control levels at 42 h and time points thereafter. In the second group, composed of *pabC* and *pabAB* (Fig. 2), expression increased to a level between 10- and 20-fold over that of the control by 36 h. The rest of the structural genes fell into the third group (Fig. 2; see Fig. S8 in the supplemental material), which demonstrated a marked level of induction by 36 h, ranging between 60-fold and 90-fold relative to the control sample. In conclusion, the expression of structural genes of the FR-008 pathway was activated by 36 h, although the degree of induction varied dramatically, suggesting that the products of these genes are required at different levels during the biosynthesis of candicidin.

Transcriptional analysis of *fscR2* and *fscR3*. Of the four regulatory genes, the transcriptional start points (TSPs) for *fscR1* and *fscR4* have been previously mapped (34). To locate the TSPs for *fscR2* and *fscR3*, we performed 5′ rapid amplification of cDNA ends (RACE) analysis. A single TSP was mapped to an adenine at position −8 relative to the *fscR3* start codon (Fig. 3). We did not detect a separate TSP for *fscR2*, possibly due to its lower transcript level, compared to that of *fscR3*, under the conditions tested or potentially from technical difficulties arising from the specific RNA architecture associated with this region. However, subsequent complementation studies with *fscR2* and its upstream intergenic region suggested that *fscR2* may have its own promoter in this region.

***fscR2* and *fscR3* are essential for candicidin production.** Of the four regulatory genes, *fscR1* and *fscR4* have been determined to be essential for normal levels of candicidin biosynthesis (34). To investigate the roles of *fscR2* and *fscR3* in candicidin biosynthesis, gene replacement mutants were generated. The *fscR2* mutant strain (Δ*fscR2*) was obtained by deleting 2,643 bp of the 2,829-bp coding sequence, corresponding to aa 30 to 910, which contains most of the AAA-ATPase domain and HTH domain. The replacement of this region by an apramycin cassette was verified by PCR analysis (see Fig. S9 in the supplemental material).

A bioassay was performed to test production of candicidin by the Δ*fscR2* strain using *Rhodotorula* as the indicator strain. While a large clear zone of inhibition was observed for the wild-type FR-008 strain (Fig. 4B), no growth inhibition of the indicator yeast was detected with the Δ*fscR2* strain (Fig. 4C), indicating that deletion of *fscR2* abrogated

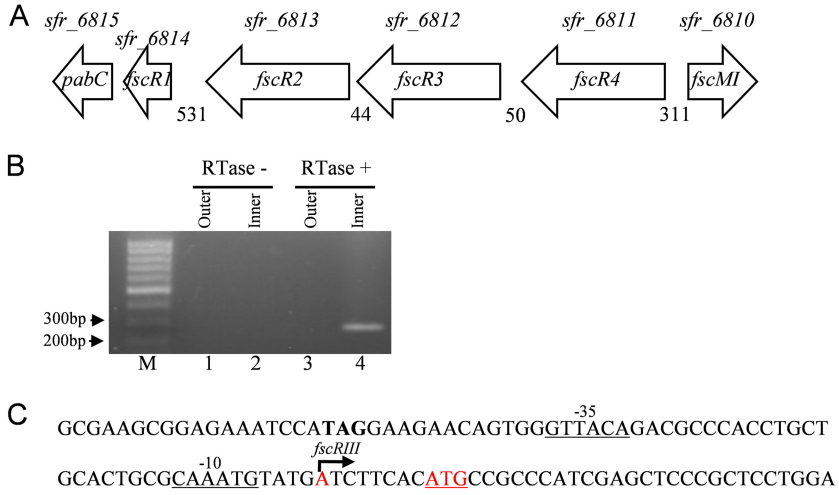

FIG 3 Location of the transcriptional start point (TSP) of *fscR3*. (A) Genetic organization of the four regulatory genes, *fscR1* to *fscR4*, and their flanking genes. Numbers indicate the lengths of the putative intergenic sequences. Gene designations shown above the arrows were obtained from the *Streptomyces* sp. FR-008 genome sequencing project (28). (B and C) Identification of the TSP of *fscR3*. (B) TSP localization by 5′-RACE. RNA was incubated without (lanes 1 and 2) or with (lanes 3 and 4) reverse transcriptase (RTase), and the cDNA was ligated to an adaptor. The cDNA ends were amplified using an outer set and then an inner set of primers, and the amplified product was sequenced to identify the TSP. M, 100-bp ladder. (C) Location of the TSP in the *fscR3* promoter. The TSP is shown in red type marked by a bent arrow. The predicted *fscR3* start codon is shown in underlined red type. The putative −10 and −35 sequences are underlined.

candicidin production. We further analyzed the level of candicidin by high-performance liquid chromatography (HPLC) analysis. Five major peaks were detected in the stock solution of candicidin (Fig. 4A), and the same peaks corresponding to the candicidin complex were detected in the extracts of the wild-type strain (Fig. 4B). However, no such peaks were detectable in the Δ*fscR2* extracts (Fig. 4C), consistent with the bioassay analysis. These data indicated that *fscR2* is critical for candicidin biosynthesis.

To confirm a direct relation between the deletion of *fscR2* and the defect in candicidin production by the Δ*fscR2* strain, a DNA segment with the coding sequence of *fscR2* and its upstream and downstream intergenic regions was cloned into pMS82, an integrative and conjugative plasmid (52), so that expression of *fscR2* would be driven by its native promoter, if present in the upstream region. The resulting plasmid, pCom-*fscR2*, was introduced into the Δ*fscR2* strain to obtain the complemented Δ*fscR2*-Com strain. The Δ*fscR2*-ComCk strain, derived by transforming the Δ*fscR2* strain with the pMS82 vector alone, was used as a control. The five peaks corresponding to the candicidin complex were detected, and a large clear zone of inhibition was restored in the Δ*fscR2*-Com strain (Fig. 4D) but not in the Δ*fscR2*-ComCk strain (Fig. 4E), confirming that FscR2 is directly involved in the regulation of candicidin biosynthesis.

Similarly, a 2,664-bp region of the 3,030-bp coding sequence of *fscR3*, corresponding to aa 99 to 986 and covering the AAA-ATPase domain, DNA polymerase III domain, and HTH domain, was replaced by an apramycin cassette to generate the Δ*fscR3* strain (see Fig. S10 in the supplemental material). Our data from by both the bioassay and HPLC analysis showed that *fscR3* is critically required for candicidin production (Fig. 4F). A direct link was established between production of candicidin and the presence of the *fscR3* gene by complementation analysis (Fig. 4G and H). These data demonstrated that both FscR2 and FscR3 are essential for candicidin production, as was previously found for FscR1 (34).

We also generated the Δ*fscR2* Δ*fscR3* mutant strain by deletion of both *fscR2* and *fscR3* and the Δ*fscR1* Δ*fscR2* Δ*fscR3* Δ*fscR4* strain, which has all four regulatory genes deleted, with each strain confirmed by PCR analysis (see Fig. S11 and S12 in the supplemental material). As expected, these two multiple-mutation strains did not

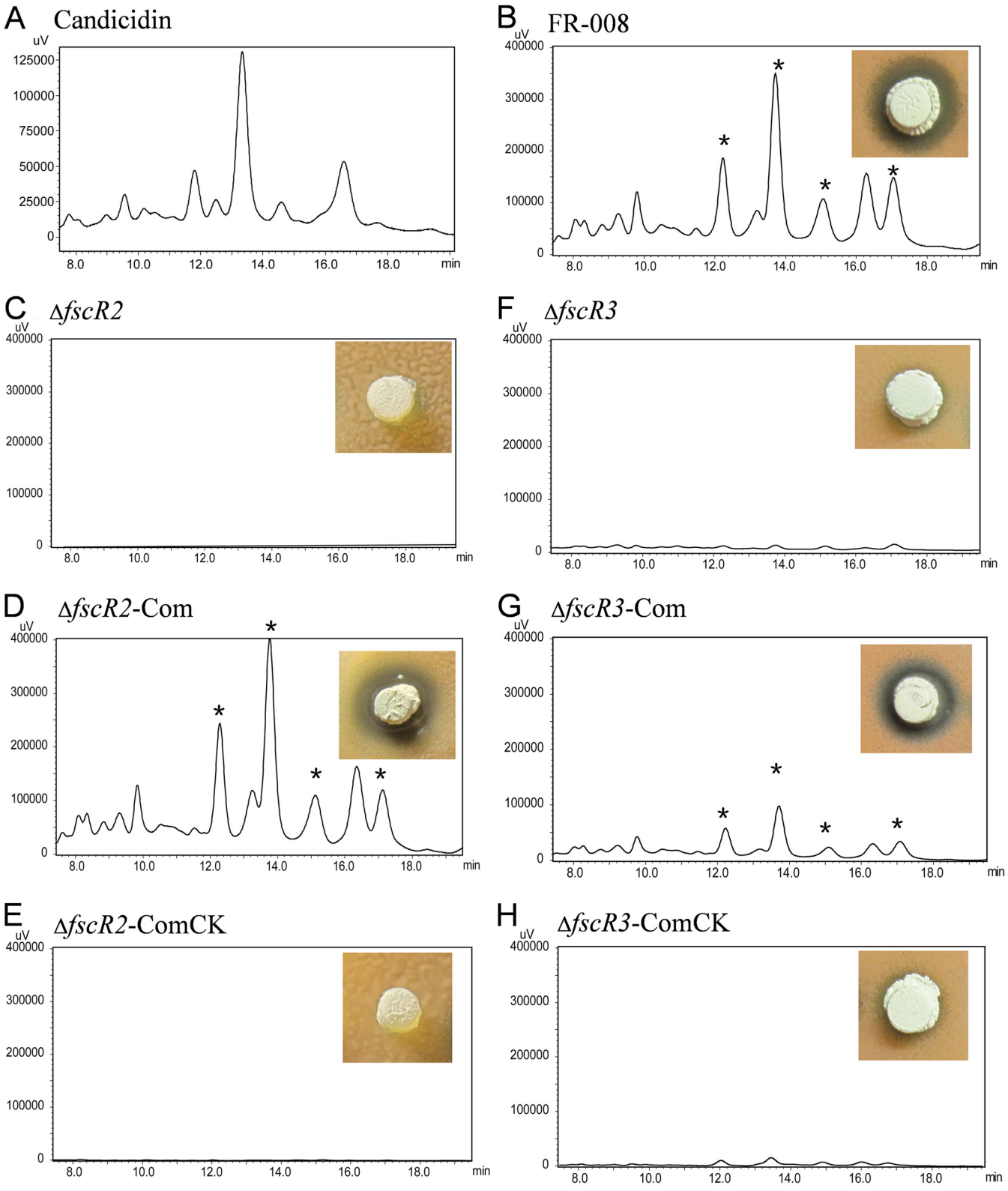

FIG 4 Production of candicidin by mutant and control strains. (A) Candicidin standard; (B) wild-type *Streptomyces* sp. strain FR-008; (C) Δ*fscR2* strain; (D) Δ*fscR2*-Com (Δ*fscR2* complemented with *fscR2*) strain; (E) Δ*fscR2*-ComCk (Δ*fscR2* complemented with vector) strain; (F) Δ*fscR3* strain; (G) Δ*fscR3*-Com (complemented with *fscR3*) strain; (H) Δ*fscR3*-ComCk (Δ*fscR3* complemented with vector) strain. The candicidin complex was extracted from strains grown on GS agar medium for 3 days before HPLC analysis. The inset photographs show the growth inhibition of the indicator yeast *Rhodotorula rubra* by each *Streptomyces* strain grown on GS agar medium for 3 days. The clear blue zone indicates growth inhibition and thus production of the candicidin complex. *, peaks corresponding to the candicidin complex.

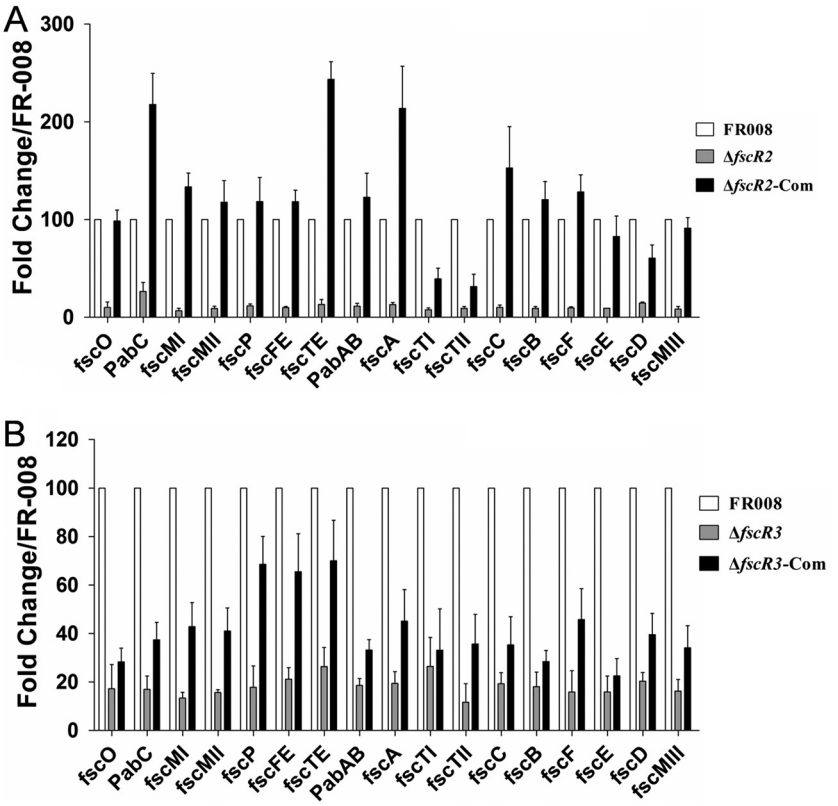

FIG 5 Transcriptional analyses of structural genes in the FR-008 pathway. (A) Expression in strain FR-008, the Δ*fscR2* strain, and the Δ*fscR2* strain complemented with *fscR2*. (B) Expression in strain FR-008, the Δ*fscR3* strain, and the Δ*fscR3* strain complemented with *fscR3*. RNA samples were isolated from strains grown for 3 days in YEME liquid medium. Expression of *hrdB*, which encodes the major sigma factor, was used as an internal control. The expression level of each gene in the wild-type strain was arbitrarily set to 100. The *y* axis shows the fold change in expression level relative to the control. Results are the means (±SD) from six biological experiments.

produce candicidin (see Fig. 8). We noticed that both the single- and the multiple-mutation strains displayed growth curves similar to that of the wild-type strain (Fig. 1A), and they also exhibited normal morphology (data not shown), suggesting that the four regulatory genes have no role in growth and differentiation.

Reduced expression of structural genes in the Δ*fscR2* and Δ*fscR3* strains. Our previous study indicated that inactivation of *fscR1* or *fscR4* leads to decreased transcription of structural genes of the FR-008 pathway. To investigate the changes in expression levels caused by deletion of *fscR2*, we performed real-time PCR analysis using RNA extracted at the 72-h time point from the Δ*fscR2* strain, the wild-type strain FR-008, and the complemented Δ*fscR2*-Com strain, which produces candicidin. The level of expression for each gene in the wild-type strain was arbitrarily set to 100 (Fig. 5A). Eleven structural genes in the Δ*fscR2* strain had expression levels of around 10 or less, i.e., less than or equal to 1/10 of control levels: *fscO* (9.9 ± 5.6), *fscMI* (6.6 ± 2.6), *fscMII* (8.9 ± 2.3), *fscMIII* (8.3 ± 2.6), *fscFE* (9.9 ± 1.1), *fscTI* (7.6 ± 1.8), *fscTII* (9.3 ± 1.8), *fscC* (10.0 ± 2.4), *fscB* (9.0 ± 1.9), *fscF* (9.5 ± 1.1), and *fscE* (9.2 ± 0.1). Five genes had expression levels of slightly above 10, i.e., over 1/10 of control levels: *fscP* (11.6 ± 1.9), *fscTE* (13.1 ± 5.1), *pabAB* (11.3 ± 2.9), *fscA* (12.9 ± 2.2), and *fscD* (14.4 ± 1.2). *pabC* had the highest expression at 26.4 ± 9.2, about one-fifth of the level in the wild-type strain. These data indicated that transcription of structural genes of the FR-008 pathway is markedly decreased in the Δ*fscR2* strain, to about 10 times lower for most of the genes. These low expression levels are consistent with the absence of detectable synthesis of candicidin under the conditions tested.

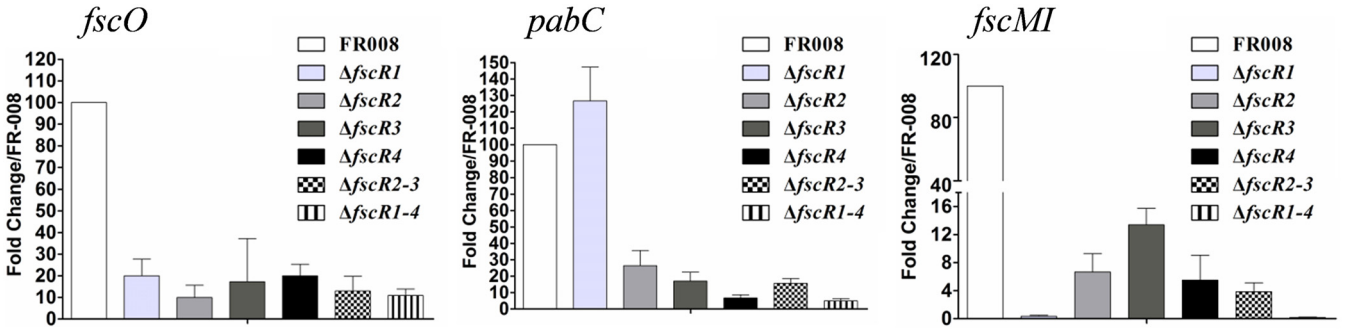

FIG 6 *fscR1* to *fscR4* exert different levels of control over structural genes in the FR-008 pathway. RNA samples were isolated from *Streptomyces* sp. FR-008, single-mutation strains, and multiple-mutation strains grown for 3 days in YEME liquid medium. Expression of *hrdB*, which encodes the major sigma factor, was used as an internal control. The expression level of each gene in the wild-type strain was arbitrarily set to 100. The *y* axis shows the fold change in expression level relative to the control. Results are the means (±SD) from six biological experiments.

We also determined the expression levels of structural genes in the Δ*fscR3* strain, with the expression of each gene in the wild-type strain set to 100. In contrast to the case for the Δ*fscR2* strain, levels lower than 10, relative to the control, were not detected for any structural gene in the Δ*fscR3* strain. The expression levels of 13 genes were between 10 and 20: *fscO* (17.2 ± 9.9), *pabC* (16.9 ± 5.4), *fscMI* (13.4 ± 2.3), *fscMII* (15.6 ± 1.2), *fscMIII* (16.2 ± 4.7), *fscP* (17.8 ± 8.8), *pabAB* (18.6 ± 2.8), *fscA* (19.4 ± 4.8), *fscTII* (11.6 ± 7.6), *fscC* (19.3 ± 4.4), *fscB* (18.1 ± 5.9), *fscF* (15.9 ± 8.7), and *fscE* (15.9 ± 6.5). Four genes had expression levels above 20: *fscFE* (21.1 ± 4.8), *fscTE* (26.4 ± 7.7), *fscTI* (26.5 ± 11.9), and *fscD* (20.4 ± 3.5). The relatively high expression levels of these structural genes, compared to the levels in the Δ*fscR2* strain, explain the slightly detectable candicidin activity of the Δ*fscR3* strain (Fig. 4F).

We also examined the expression levels of structural genes in the two complemented Δ*fscR2*-Com and Δ*fscR3*-Com strains (Fig. 5). In the Δ*fscR2*-Com strain, expression of three structural genes (*fscO*, *fscE*, and *fscMIII*) was restored to levels comparable to those in the wild-type strain, 11 genes (*pabC*, *fscMI*, *fscMII*, *fscP*, *fscFE*, *fscTE*, *pabAB*, *fscA*, *fscC*, *fscB*, and *fscF*) were expressed at levels higher than those in the wild-type control, and only three genes (*fscTI*, *fscTII*, and *fscD*) had expression lower than that in the control, consistent with the near-normal levels of bioactivity for the Δ*fscR2*-Com strain (Fig. 4C; see Fig. 8). In contrast, in the Δ*fscR3*-Com strain, only three genes had expression levels restored to between 60 and 70, with all the rest having levels below 50 (Fig. 5B), relative to controls, consistent with the moderately restored bioactivity for this strain (Fig. 4G; see Fig. 8) and further supporting a direct correlation between the relative bioactivity levels among the strains and the comparative expression levels of their structural genes. Altogether, our data indicated that the expression of structural genes in the FR-008 pathway was decreased in both the Δ*fscR2* and Δ*fscR3* strains, but the levels of reduction varied.

The four regulatory genes exert different levels of control over structural genes. The different transcriptional levels of structural genes in the Δ*fscR2* and Δ*fscR3* strains reflect different degrees of control over these genes by the regulatory genes. To compare the relative levels of control of the structural genes by each regulatory gene, we performed real-time PCR analysis using our mutant strains for each of the four regulatory genes and arbitrarily set the expression level of each gene in the wild-type FR-008 strain to 100 (Fig. 6; see Fig. S13 in the supplemental material). Based on the data, structural genes could be divided into three groups. *fscO* is the only gene in group 1, with an expression level ranging between 10 and 20, relative to the control level of 100, in all four single mutant strains (Δ*fscR1*, Δ*fscR2*, Δ*fscR3*, and Δ*fscR4*), indicating similar levels of control of this gene by the four regulatory genes. Group 2 contains only *pabC*, whose level in the Δ*fscR1* strain (126 ± 26) was even higher than that in the wild-type strain, while its level in the other three single mutants ranged from 6 to 26, indicating negative control of *pabC* by FscR1 in contrast to positive control by FscR2,

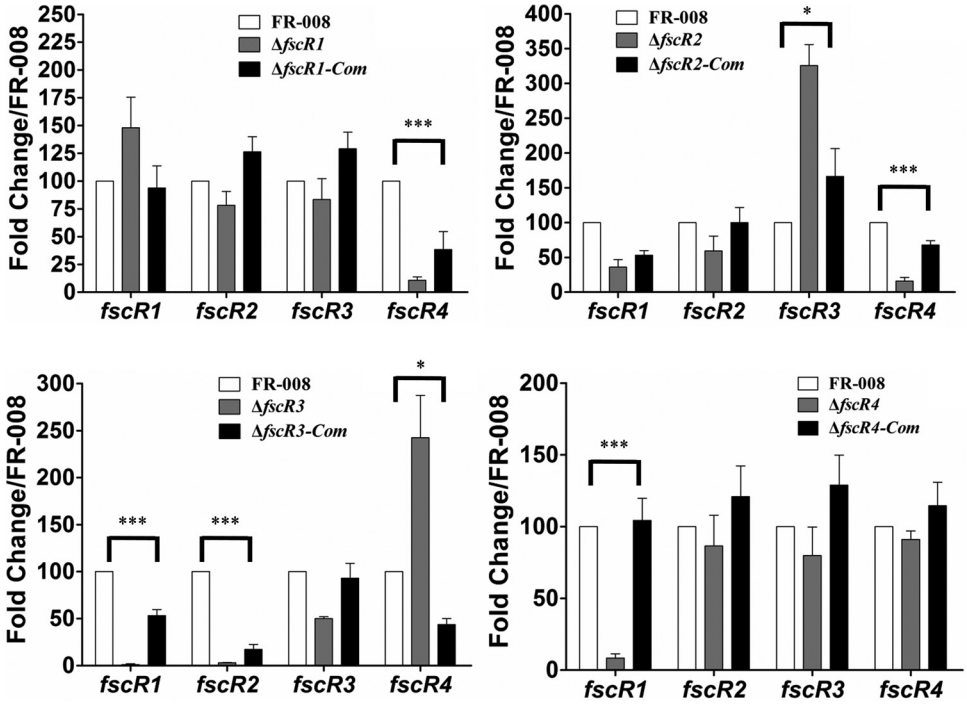

FIG 7 Cross-regulation between regulatory genes. The same RNA samples were used as for Fig. 6. The expression level of each gene in the wild-type strain was arbitrarily set to 100. The *y* axis shows the fold change in expression level relative to the control. Primers for the four regulatory genes were designed to a portion of the coding sequence that was not deleted from the respective mutant. Results are the means (±SD) from six biological experiments. *, $P < 0.05$; ***, $P < 0.01$.

FscR3, and FscR4. All the other structural genes are in group 3 (Fig. 6 and S13), in which the expression level of genes in the Δ*fscR1* strain was below 1 and expression in the other single mutants varied, but with all levels much lower than in the wild-type strain. Additionally, for group 3, the expression levels in the Δ*fscR3* strain were often higher than those in the Δ*fscR2* and Δ*fscR4* strains, and the expression in the Δ*fscR2* strain was generally higher than in the Δ*fscR4* strain, indicating overall that FscR1 has the tightest control over these structural genes, followed by FscR2, FscR4, and then FscR3.

We also evaluated the expression levels of genes in the Δ*fscR2* Δ*fscR3* and Δ*fscR1* Δ*fscR2* Δ*fscR3* Δ*fscR4* multiple-mutation strains. Expression in the Δ*fscR2* Δ*fscR3* double mutant strain was always lower than that in the Δ*fscR2* and Δ*fscR3* single mutant strains, and expression in the Δ*fscR1* Δ*fscR2* Δ*fscR3* Δ*fscR4* quadruple mutant strain was the lowest and was barely detectable for most genes (Fig. 6 and S13), suggesting a combined effect of the gene deletions.

Cross-regulation between the four regulatory genes. Using the four single mutant strains, we examined the effects of the regulatory genes on each other by real-time PCR analysis using primers specific to the undeleted portion of each regulatory gene (Fig. 7). *hrdB* was used as an internal control, and the expression level of each gene in the wild-type strain was arbitrarily set to 100 and was used as a control. Transcription of *fscR1* was upregulated slightly in the Δ*fscR1* strain, indicating a minor autorepressive effect; however, expression of *fscR2* and *fscR3* in the Δ*fscR1* strain was comparable to levels in the wild-type strain, indicating that FscR1 does not impact transcription of *fscR2* and *fscR3*. In contrast, expression of *fscR4* was only 10 ± 2, about 1/10 of the wild-type level, indicating a significantly positive regulatory effect on *fscR4* by FscR1, in agreement with our previous report (34). Our data showed that transcription of two regulatory genes was significantly affected by mutation of *fscR2*; expression of *fscR3* (325 ± 29) was significantly enhanced, whereas *fscR4* expression (15 ± 5) was significantly reduced in the Δ*fscR2* strain relative to the control, indicating that FscR2

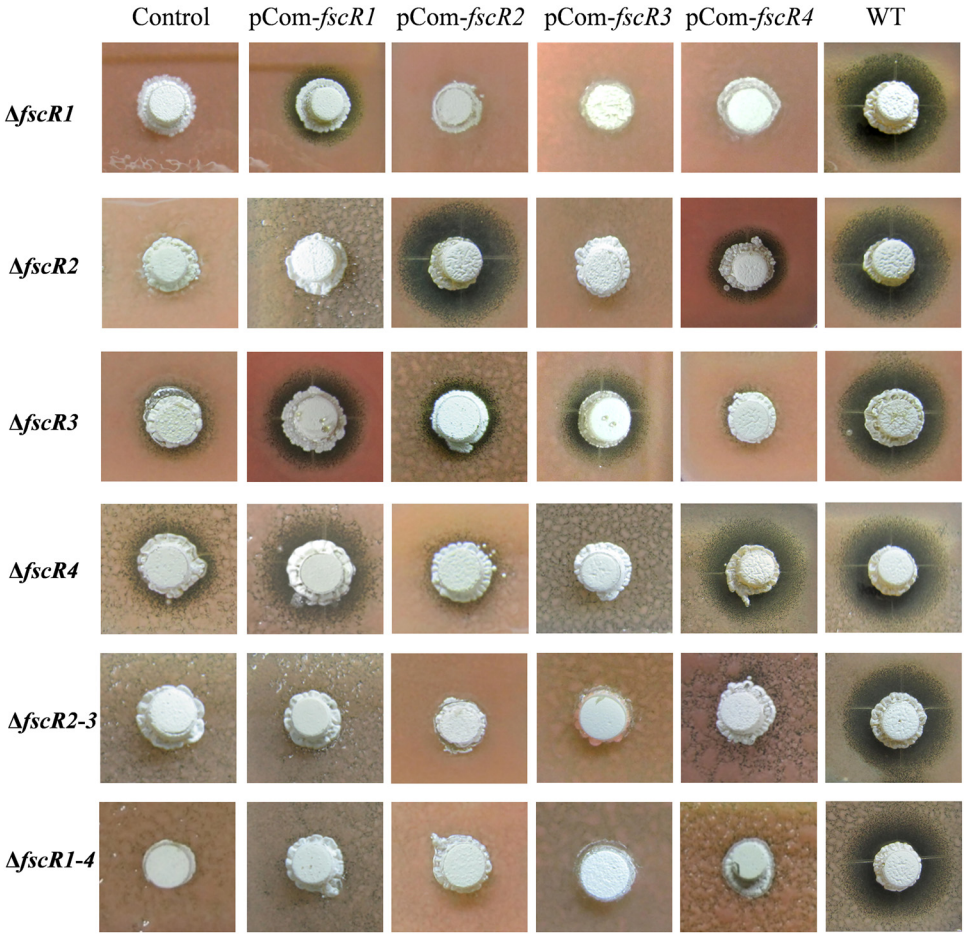

FIG 8 Cross-complementation between regulatory genes. Complementation plasmids (pCom-*fscR1* through pCom-*fscR4*) for each of the four regulatory genes were introduced into each single- and multiple-mutation strain, and the resulting strains were tested for production of candicidin by bioassay after growth for 3 days on MS agar medium. Control column, mutant strains complemented with the vector only; WT, wild-type FR-008 strain.

negatively regulates *fscR3* expression but positively regulates *fscR4* expression. Basal transcription levels of 1.1 ± 0.9 and 3.0 ± 0.4 were detected for *fscR1* and *fscR2*, respectively, in the Δ*fscR3* strain, indicating a profound positive regulatory effect of FscR3 on these two genes. In contrast, markedly enhanced transcription (242 ± 45) was detected for *fscR4* in the Δ*fscR3* strain (Fig. 7), indicating a significantly negative regulatory effect on this gene by FscR3. In the Δ*fscR4* strain, only transcription of *fscR1* was significantly impacted, with a decrease to 8.4 ± 2.9, indicating strong regulation of this gene by FscR4, in agreement with our previous report (34). In conclusion, each of the four regulatory factors demonstrated marked regulation over at least one regulatory gene, with both positive and negative effects observed, forming a complex regulatory network.

Cross-complementation between the four regulatory genes *fscR1* to *fscR4*. The cross-regulation between the four regulatory genes, *fscR1* to *fscR4*, prompted us to investigate potential functional substitution between these genes. Therefore, we performed a comprehensive cross-complementation analysis by introducing complementation plasmids that contain one copy of each regulatory gene driven by its native promoter into each single mutant and tested candicidin production by each transformant by bioassay (Fig. 8) and by HPLC (see Fig. S14 to S19 in the supplemental material). Our data indicated that *fscR1* partially restored candicidin production to the Δ*fscR3* and Δ*fscR4* strains, indicating functional substitution of FscR3 and FscR4 by FscR1. Similarly, *fscR4* restored some candicidin production to the Δ*fscR2* strain, indi-

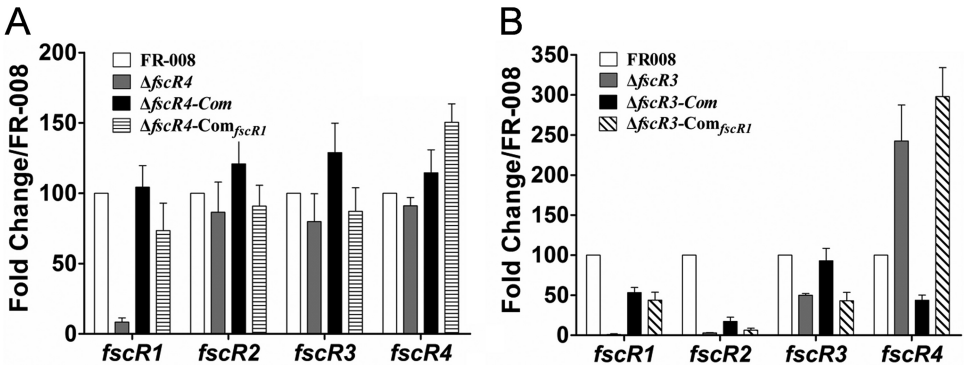

FIG 9 Transcription of regulatory genes in heterocomplemented strains. RNA samples were isolated from *Streptomyces* sp. FR-008, the *fscR3* and *fscR4* mutants, and complemented strains grown for 3 days in YEME liquid medium. Expression of *hrdB*, which encodes the major sigma factor, was used as an internal control. The expression level of each gene in the wild-type strain was arbitrarily set to 100. The *y* axis shows the fold change in expression level relative to the control. Results are the means (±SD) from six biological experiments. Primers for these genes were designed to an undeleted portion of the coding sequence. Δ*fscR3*-Com$_{fscR1}$ and Δ*fscR4*-Com$_{fscR1}$, Δ*fscR3* and Δ*fscR4* complemented with *fscR1*.

cating that FscR4 can at least partially functionally substitute for FscR2. Notably, the functional substitution (heterocomplementation) appears to be unidirectional. Whereas *fscR1* was able to complement the Δ*fscR3* and Δ*fscR4* strains, neither *fscR3* nor *fscR4* could complement the Δ*fscR1* strain (Fig. 8). Similarly, *fscR4* was able to complement the *fscR2* deletion, but not vice versa, suggesting a regulatory hierarchy for these genes.

The four complementation plasmids were also transformed into the Δ*fscR2* Δ*fscR3* double mutant strain and the Δ*fscR1* Δ*fscR2* Δ*fscR3* Δ*fscR4* quadruple mutant strain; however, none was able to restore candicidin production to these multiple-mutation strains (Fig. 8). Overall, the findings suggest that optimal production of candicidin requires the presence and coordinated action of all four regulatory genes.

Expression of *fscR1* is restored in functionally heterocomplemented strains. Functional heterocomplementation of the Δ*fscR3* and Δ*fscR4* strains was detected with *fscR1*, which restored candicidin production to these two mutant strains (Fig. 8 and S14 to S19). To investigate the mechanisms underlying this restoration in the Δ*fscR4* strain, we compared the transcription of genes in the FR-008 pathway using the wild-type FR-008 and the Δ*fscR4*, Δ*fscR4*-Com (Δ*fscR4* complemented by *fscR4* itself), and Δ*fscR4*-Com$_{fscR1}$ (Δ*fscR4* complemented by *fscR1*) strains (Fig. 9A). With the expression of *fscR1* in the wild-type strain set at 100, the expression of *fscR1* in the Δ*fscR4* strain was only 8.4 ± 2.9; however, it was restored to 74 ± 19 in the Δ*fscR4*-Com$_{fscR1}$ strain, nearly three-quarters of the full expression level. We also noticed that the expression level of *fscR4* in the Δ*fscR4*-Com$_{fscR1}$ strain was even slightly higher than that in the Δ*fscR4*-Com strain, supporting a positive effect of FscR1 on *fscR4*. In contrast, complementation of Δ*fscR4* with *fscR1* did not have any obvious effects on the expression of *fscR2* and *fscR3* (Fig. 9A), consistent with our previous data indicating that FscR1 does not impact *fscR2* and *fscR3* expression (Fig. 7). We also examined the transcription of structural genes of the FR-008 pathway in the Δ*fscR4*-Com$_{fscR1}$ strain (Fig. 10A). In general, the markedly lower expression of these genes in the Δ*fscR4* strain was restored to levels comparable to those in the wild-type strain in the Δ*fscR4*-Com$_{fscR1}$ strain, indicating that *fscR1* is responsible for the basal transcription of the structural genes and that the defect in candicidin biosynthesis in the Δ*fscR4* strain is largely due to reduced *fscR1* transcription.

Similarly, we performed transcription analysis in the Δ*fscR3*-Com$_{fscR1}$ strain (Δ*fscR3* complemented with *fscR1*) (Fig. 9B). Expression of *fscR1* was barely detectable in the Δ*fscR3* strain, but was restored to a relative level of 44 ± 9 in the Δ*fscR3*-Com$_{fscR1}$ strain, comparable to the level (53 ± 6) in the Δ*fscR3*-Com strain (Δ*fscR3* complemented with *fscR3* itself), although both levels were lower than that in the control strain. The expression of the other three regulatory genes was not altered in the Δ*fscR3*-Com$_{fscR1}$ strain, suggesting that FscR1 does not impact these genes under this condition. The

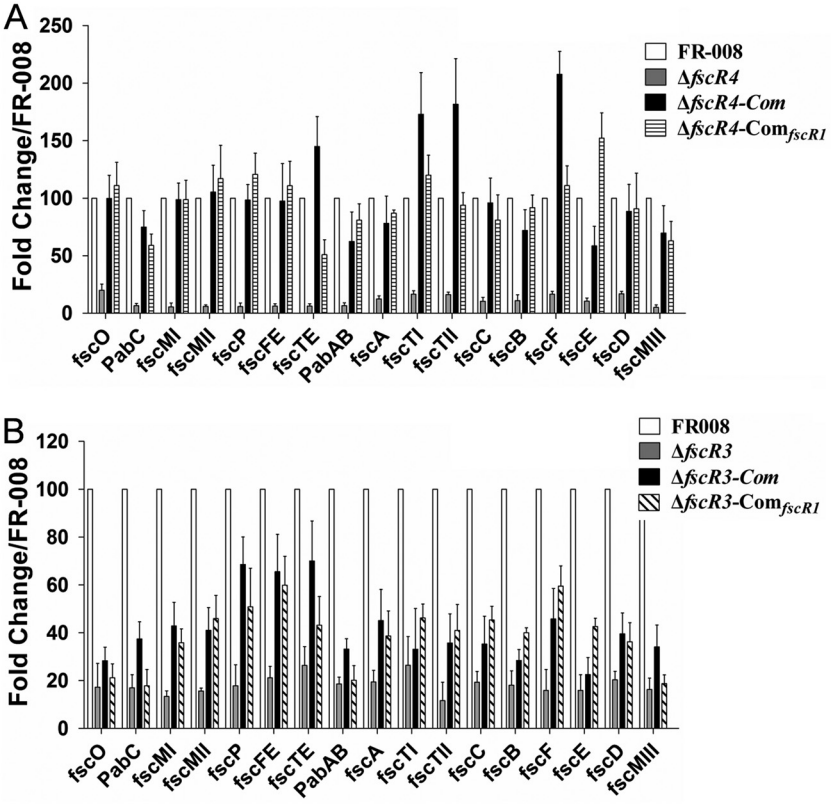

FIG 10 Transcription of structural genes in heterocomplemented strains. RNA samples were isolated from *Streptomyces* sp. FR-008, the *fscR3* and *fscR4* mutants, and complemented strains grown 3 days in YEME liquid medium. Expression of *hrdB*, which encodes the major sigma factor, was used as an internal control. The expression level of each gene in the wild-type strain was arbitrarily set to 100. The *y* axis shows the fold change in expression level relative to the control. Results are the means (±SD) from six biological experiments. Δ*fscR3*-Com$_{fscR1}$ and Δ*fscR4*-Com$_{fscR1}$, Δ*fscR3* and Δ*fscR4* complemented with *fscR1*.

expression of most structural genes of the FR-008 pathway in the Δ*fscR3*-Com$_{fscR1}$ strain was similar to that in the Δ*fscR3*-Com strain (Fig. 10B), although both complemented strains had expression levels lower than those in the wild-type control, explaining the partial restoration of candicidin production. In conclusion, the reduced expression level of *fscR1* appears to be the main factor in the defect in candicidin biosynthesis in the Δ*fscR3* and Δ*fscR4* strains.

Functional heterocomplementation was also detected between *fscR4* and Δ*fscR2* (Fig. 8 and S14). Our transcriptional analysis indicated that introduction of *fscR4* into the Δ*fscR2* strain restored expression of *fscR4* itself and of *fscR1*, but not of that of *fscR2* or *fscR3*, to levels comparable to those in the Δ*fscR2*-Com strain (see Fig. S20A in the supplemental material), supporting our results indicating that FscR4 activates *fscR1* (Fig. 7). Consistent with the upregulation of *fscR1*, the expression of structural genes in the Δ*fscR2*-Com$_{fscR4}$ strain was partially restored (Fig. S20B), resulting in detectable production of candicidin (Fig. 8 and S14 to S19).

We also investigated the mechanisms by which heterocomplementation failed, in one pairing, by examining the transcription of genes in the Δ*fscR1* strain complemented with *fscR4*, which is downregulated in the Δ*fscR1* strain (Fig. 7). Although the Δ*fscR1* strain does not contain a functional *fscR1* gene, primers were designed to detect transcription of the small remaining portion of the gene. Our transcriptional analysis indicated that introduction of an extra copy of *fscR4* into the Δ*fscR1* strain did not restore the expression of *fscR1* or of *fscR4* itself (see Fig. S21A in the supplemental material), indicating that expression of *fscR4* needs a functional FscR1. Additionally, expression of the structural genes was not restored in the Δ*fscR1*-Com$_{fscR4}$ strain (Fig.

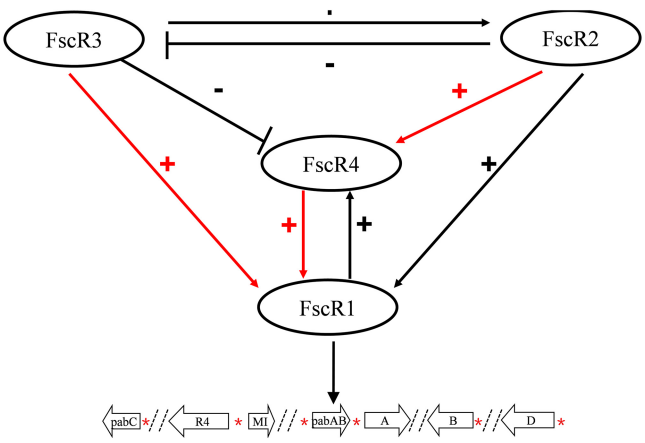

FIG 11 Model of the regulatory network formed by the four regulatory proteins, FscR1 to FscR4, controlling production of the polyene antibiotic candicidin in *Streptomyces* sp. FR-008. FscR2 and FscR3 are located at the upper level of the network; FscR3 positively regulates *fscR2*, but FscR2 negatively regulates *fscR3*, forming a negative feedback loop. The two cross-regulating factors FscR1 and FscR4 are at the lower levels of the regulatory hierarchy, with *fscR1* positively regulated by both FscR2 and FscR3 and *fscR4* regulated positively by FscR2 but negatively by FscR3. FscR1 mediates the regulatory effect of the other three regulatory factors by directly regulating the structural genes. +, positive regulatory effect; −, negative regulatory effect. A red arrow indicates functional substitution of the upstream gene by the downstream gene, i.e., by the gene at the lower level of the hierarchy. *, locations of FscRI recognition sites in the promoters of the structural genes.

S21B), consistent with the absence of functional complementation by *fscR4* and our evidence that FscR1 is the key regulatory factor involved in activation of these structural genes.

Proposed regulatory network of the four regulatory genes, *fscR1* to *fscR4*, controlling production of the polyene antibiotic candicidin. Based on our data, we propose a model of the regulatory network comprising *fscR1* to *fscR4* (Fig. 11). FscR2 and FscR3 are located at the top of the regulatory hierarchy, with FscR3 positively regulating *fscR2* but FscR2 negatively regulating *fscR3*, forming a negative feedback loop. At the next level, FscR1 and FscR4 positively regulate each other, with *fscR1* also regulated positively by both FscR2 and FscR3. *fscR4* is regulated positively by FscR2 but negatively by FscR3. The functional substitution of *fscR1* for *fscR3* and *fscR4*, and of *fscR4* for *fscR2*, is consistent with the model that *fscR1* functions downstream of FscR3 and FscR4 and *fscR4* functions downstream of FscR2. FscR1 functions at the lowest tier of the hierarchy, where it mediates the regulatory effect of the other three regulators by directly controlling expression of the structural genes for candicidin biosynthesis (34).

DISCUSSION

One unique feature of the candicidin pathway and other polyene antibiotic pathways is the regulatory subcluster containing four consecutive regulatory genes, which encode transcriptional factors belonging to two different families. The data from this study and a previous study by our group showed that each of these regulatory genes contributes to the optimal production of candicidin by *Streptomyces* sp. FR-008 (34). The key roles of these regulatory genes in the production of polyene antibiotics are supported by the requirement for their orthologues for nystatin biosynthesis in *Streptomyces noursei* ATCC 11455 (36).

PAS-LuxR regulators such as FscR1 have been implicated in multiple cellular processes, and even when they are located within a specific gene cluster, they may regulate other pathways (45, 53). FscR1 also regulates production of antimycin (45), although in this study, we focused on the relationship of FscR1 and the other three cluster-associated regulators with production of candicidin. Mutation of the four regulatory genes did not affect growth, morphology, or tested physiological activities other than production of candicidin. Interestingly, these genes displayed similar tem-

poral expression patterns, with all of them activated at an early growth stage during which production of candicidin is initiated. The structural genes of the FR-008 pathway also demonstrated temporal expression patterns similar to those of the four regulatory genes, suggesting coordinated expression of these four regulatory genes in activation of the structural genes and therefore production of the antibiotic. However, surprisingly, the relative expression levels of these four regulatory genes varied to a large extent (Fig. 1D), from the most highly expressed gene, *fscR1*, to *fscR4*, which was expressed at a 100-fold-lower level, indicating differential expression for these four genes during growth and suggesting a dominant role for FscR1 in activation of the pathway.

In *Streptomyces* species, production of an antibiotic is usually coupled with cell differentiation on solid medium at later growth phases (41, 42). However, in our study, both regulatory genes and structural genes of the FR-008 pathway were activated by 36 h, and candicidin production was evident by this relatively early time. These results suggest that *Streptomyces* FR-008 may be more vulnerable to competition from fungal agents during the early stages of growth, and hence, candicidin is produced earlier than other antibiotics. Additionally, the fast growth of strain FR-008 may also contribute to the early production of antibiotic (28).

Each of the four regulatory genes is required for candicidin biosynthesis, and deletion or mutation of each of them led to reduced expression of the structural genes and, consequently, a defect in candicidin biosynthesis. However, the levels of reduced expression of the structural genes varied among the mutants. The expression of most structural genes decreased by more than 100 times in the Δ*fscR1* mutant compared with the wild-type control, explaining the complete abrogation of candicidin production by the Δ*fscR1* strain. Although the expression of most structural genes was higher in the Δ*fscR2* strain than in the Δ*fscR1* strain, the levels were still not high enough for the Δ*fscR2* strain to produce enough candicidin compounds for detection under the conditions tested. The highest expression level of structural genes was observed in the Δ*fscR3* strain, which demonstrated leaky activity by the bioassay and minor peaks in HPLC analysis, indicating that transcription of the structural genes was just sufficient in this mutant to enable detectable candicidin synthesis. We conclude that the level of candicidin production is closely associated with the expression level of structural genes in the FR-008 pathway.

Although the evolution of the regulatory subcluster with the four regulatory genes is not clear, based on the fact that deletion of each regulatory gene blocks or nearly blocks candicidin biosynthesis, our study indicates that a finely coordinated and simultaneous expression of these four regulatory genes is essential for maintaining a normal level of candicidin biosynthesis, suggesting that the clustered arrangement may be optimal for coordinated regulation.

We revealed that each regulatory gene impacts the expression of at least one other regulatory gene, thus forming a complex regulatory network. Therefore, it should be noted that any changes in expression in a particular mutant strain may result from the combined effect of the specific mutation as well as the altered expression of the regulatory gene(s) that the mutated factor regulates. For example, in the *fscR3* mutant, the decrease in transcription of structural genes resulting from the loss of *fscR3* and reduced expression of *fscR1* and *fscR2* may be modulated by the enhanced expression of *fscR4* in this mutant (Fig. 7). In a previous study, we characterized the recognition sequence of FscR1 and revealed multiple targets of FscR1, including the first genes of several operons in the FR-008 pathway (34). However, the large size of FscR2, FscR3, and FscR4 made it difficult to express these proteins in *Escherichia coli*, and thus we did not determine whether the regulation of structural genes by these three regulatory proteins is direct or indirect. However, as *fscR1* is located downstream of FscR2, FscR3, and FscR4 in the regulatory network and FscR1 was able to functionally complement *fscR3* and *fscR4*, it is likely that *fscR1* mediates the regulatory effect of the other three regulatory genes.

Overall, we obtained substantial insight into understanding the role of, and espe-

TABLE 1 Microbial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
Streptomyces		
FR-008	Wild type	26
Δ*fscR1* strain	Deletion of internal sequence of *fscR1*, unmarked	34
Δ*fscR2* strain	*fscR2*::*aac(3)IV*	This study
Δ*fscR3* strain	*fscR3*::*aac(3)IV*	This study
Δ*fscR4* strain	Deletion of internal sequence of *fscR4*, unmarked	34
Δ*fscR2* Δ*fscR3* strain	Deletion of sequence from *fscR2* to *fscR3*, unmarked	This study
Δ*fscR1* Δ*fscR2* Δ*fscR3* Δ*fscR4* strain	Deletion of sequence from *fscR1* to *fscR4*, unmarked	This study
Δ*fscR1*-Com$_{fscR4}$ strain	Δ*fscR1* strain complemented with pCom-*fscR4*	This study
Δ*fscR2*-Com strain	Δ*fscR2* strain complemented with pCom-*fscR2*	This study
Δ*fscR2*-Com$_{fscR4}$ strain	Δ*fscR2* strain complemented with pCom-*fscR4*	This study
Δ*fscR3*-Com strain	Δ*fscR3* strain complemented with pCom-*fscR3*	This study
Δ*fscR3*-Com$_{fscR1}$ strain	Δ*fscR3* strain complemented with pCom-*fscR1*	This study
Δ*fscR4*-Com strain	Δ*fscR4* strain complemented with pCom-*fscR4*	This study
Δ*fscR4*-Com$_{fscR1}$ strain	Δ*fscR4* strain complemented with pCom-*fscR1*	This study
E. coli		
Novablue	General cloning strain	Novagen
ET12567(pUZ8002)	Strain used for conjugation between *E. coli* and *Streptomyces*	43
Rhodotorula rubra	Indicator for candicidin production	
Plasmids		
pEASY-Blunt	General cloning vector	Invitrogen
pMS82	*Streptomyces* integrative vector with hygromycin resistance	52
pCom-*fscR1*	pMS82 with 531-bp upstream sequence, 696-bp coding sequence, and 200-bp downstream sequence of *fscR1*	This study
pCom-*fscR2*	pMS82 with 579-bp upstream sequence, 2,829-bp coding sequence, and 152-bp downstream sequence of *fscR2*	This study
pCom-*fscR3*	pMS82 with 319-bp upstream sequence, 3,030-bp coding sequence, and 95-bp downstream sequence of *fscR3*	This study
pCom-*fscR4*	pMS82 with 404-bp upstream sequence, 2,913-bp coding sequence, and 200-bp downstream sequence of *fscR4*	This study

cially the regulatory network formed by, the four regulatory genes of the FR-008 pathway. We found that the four regulatory genes are differentially expressed throughout growth and that their control over structural genes varies to a large extent. We also revealed cross-regulation between the four regulatory genes, resulting in a complex regulatory network, and we found some functional heterocomplementation among these four regulatory factors, with genes at the lower level of the regulatory hierarchy restoring the loss of activity resulting from mutation of the genes at the upper level. The network formed by the four regulatory genes of the FR-008 pathway can serve as a model for exploring gene regulation in polyene and other complex antibiotic pathways.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The microbial strains and plasmids used in this study are listed in Table 1. The wild-type *Streptomyces* sp. FR-008 and its derivatives were grown on glycerol soya flour agar for spore preparation, antibiotic production, and conjugation transfer (43) and in YEME liquid medium (43) for growth curve preparation, bioassay, and RNA extraction. *Escherichia coli* strains were cultivated in Luria-Bertani (LB) liquid or solid agar medium. Antibiotics were added, if necessary, for selection of transformants from *E. coli* or *Streptomyces*.

Mutation of genes in *Streptomyces* sp. FR-008. The coding sequence of *fscR2* was replaced by an apramycin resistance cassette through homologous recombination using a strategy similar to that described previously (8, 54). Briefly, the DNA fragment serving as the left arm was amplified with RII-LF (with a HindIII site) and RII-LR (with a SpeI site). The amplified DNA fragment was purified and ligated with pEASY-Blunt to generate pRII-HL-Arm. The DNA fragment serving as the right arm was amplified with primers RII-RF (with a SpeI site) and RII-RR (with an XbaI site) and was cloned into pEASY-Blunt to generate pRII-HR-Arm. Primers Apr-Forward (with a SpeI site) and Apr-Reverse (with a SpeI site) were used to amplify a DNA fragment containing the apramycin cassette, using pIJ773 as the template, and the amplified cassette sequence was cloned into pMD-18T to generate pApr-M. pRII-HL-Arm was then digested by SpeI and XbaI (which cut in the vector sequence) and ligated with the right arm, which was released from pRII-HR-Arm by SpeI and HindIII digestion, to generate pRII-HL-R, in which the left and right arms were fused. pRII-HL-R was then cut by SpeI and ligated with the apramycin cassette, which had

TABLE 2 Primers used in this study

Category and name	Sequence (5′→3′)[a]
fscR2 mutation	
RII LF/LR	AAGCTTGGGCACACGGTGTACGGATCACTTG (HindIII)/ACTAGTAGCACCTGACCCACGCCTAC (SpeI)
RII RF/RR	ACTAGTTGAGGAGGACGAGGGAGG (SpeI)/TCTAGAACCGAACGGCACGAACTGAC (XbaI)
Apra F/R	ACTAGTGAGCTCAGCCAATCGACTGG (SpeI)/ACTAGTACAGTGCCGTTGATCGTGCTATGA (SpeI)
fscR2 mutation analysis	
RII VerF/R	CGACATTCTGGGGGACGCCGGATGT/CAGGTCCTCCCTCGTCCTCCTCACC
fscR3 mutation	
RIII LF/LR	AAGCTTTGGTGAGGGTGGACTCGG (HindIII)/ACTAGTCGTCTACCGCAAACTCCG (SpeI)
RIII RF/RR	ACTAGTTCGTCGGTGAGGAAGAGGC (SpeI)/TCTAGAGGTCTTCGGGTCCTGCG (XbaI)
fscR3 mutation analysis	
RIII VerF/R	CGAAGGCGAAGTCCTGTTCC/GAACTCCACCTCACCAGCGTC
fscR1 to *-4* mutation	
RI-RIV LF/LR	AAGCTTTGCTCCACCGCTTCCACCTT (HindIII)/ACTAGTGGGCGTCCTCAAGGTCGG (SpeI)
RI-RIV RF/RR	ACTAGTCCGACGCGAGGGAGAGCCCTGTG (SpeI)/TCTAGAGTCGAGCCACCTGGTGACCTCGTCG (XbaI)
fscR1 to *-4* mutation analysis	
RI-RIV VerF/R	AAGCCCATGTAGACCACCGACGACT/GGCGGCGATGTTCCCCAGGTTCAGC
fscR2 and *-3* mutation	
RII-RIII LF/LR	AAGCTTCGAGCAGCGTGGTGAAATG (HindIII)/CTCGAGAGCACCTGACCCACGCCTAC (XhoI)
RII-RIII RF/RR	CTCGAGCATGTGAAGATCATACATTTGC (XhoI)/TCTAGACACAGCGGTTCAGCTGGGCCTGACC (XbaI)
fscR2 and *-3* mutation analysis	
RII-RIII VerF/R	GCGCTGGAGATCCTCGGCCAGGA/TGGTGACGAACGTTCATCCAGAT
fscR1 complementation	
RI-MS82-F/R	GAGAACCTAGGATCCAAGCTTTACGCGCCGAGGGAAACG/ACCATGCATAGATCTAAGCTTCGAGCAGCGTGGTGAAATGGCCG
fscR2 complementation	
RII-MS82-F/R	GAGAACCTAGGATCCAAGCTTCGCCCGGCTGGTCCACGAGGAAGT/ACCATGCATAGATCTAAGCTTGAACGTTCATCCAGATTTCCCGGC
fscR3 complementation	
RIII-MS82-F/R	GAGAACCTAGGATCCAAGCTTGTCACCGCCTCACCGCTGGACATGCT/ACCATGCATAGATCTAAGCTTCGGTGAGGAGGACGAGGGAGGAC
fscR4 complementation	
RIV-MS82-F/R	GAGAACCTAGGATCCAAGCTTCCGGGCAAGCTCCCCGGCCAGCA/ACCATGCATAGATCTAAGCTTGCAGCAGATCGTTCTGGCCGAGCCC
Rapid amplification of cDNA ends	
RIII 5-RACE	TCAGGGGGGTGCCGACGCTCCACT/ACGCCGTACCGCACCTCGTGTTC
RII 5-RACE Outer	GAGGATGAGCAGGCGGCT/AAGAGCGGGTTGCCGACGGA
RII 5-RACE Inner	GCGTCTCGAAGAGCTGCCGT/CCGAAGGCGAAGTCCTGTT

(Continued on next page)

TABLE 2 (Continued)

Category and name	Sequence (5′→3′)[a]
Real-time PCR analysis	
sigma RT-F/R	GGGCTACAAGTTCTCCACG/AGGTCCTGGAGCATCTGGC
fscA RT-F/R	TGACCGAGATGCTGGAGCG/GTGCGAGTGGAGGCAGATC
fscB RT-F/R	CCACCACGGCACCACCTACA/AGGGTGTCGGGCAGGATG
fscC RT-F/R	GAGCCCATCGCCGTCATCG/GAAGCCGCCCTCACGGACAT
fscD RT-F/R	GACACCGCCTGCTCCTCCT/ACGAAGGTGGTGGGGCTGGC
fscE RT-F/R	GGCACCAACGGCCAGGACTA/AGGAGCAGGCGGTGTCGA
fscF RT-F/R	ATGCCCGATGACAAGAAGC/TCCAGGAGCCGCCAGTA
fscMI RT-F/R	GATGTCGGTGCGGCAGAAG/CGATGCCCAGTTCCTTGCGT
fscMII RT-F/R	GACATCGCCTGCTTCTCG/CTTGTGCAGGAAGCTGTGG
fscMIII RT-F/R	GCGGTGGAAAGAGGAAAGATG/TGAGGTCGCCGTCCACGAAG
pabC RT-F/R	CATTTCACCACGCTGCTCG/AGACGGTGACACGGACGG
fscP RT-F/R	GAGCACGACCGTTTCCGC/CGAAGTCGTTCACCAGGTCC
fscO RT-F/R	CCCTTCCGGGCCTACAA/TGCTCCACCGCTTCCAC
fscFE RT-F/R	TGCGTAGGAGCGGGCCAGTG/CTCCTGGAGGACGACCGA
fscTE RT-F/R	GCCAGAACCATCGAAGGGAC/TGGTAGAAGCTGGCGGAGCC
pabAB RT-F/R	ACCCGAGGTCATCCGCAACG/GCGCACAGGCCGAAGTCG
fscTI RT-F/R	GCGTCAGCAAGGCATACGGC/GTGGTGAGGATGTCGACCAG
fscTII RT-F/R	TGGTCATCCTCTTCGTGCTG/GATGCCGTTCTTCTGGTCC
fscRI RT-F/R	TGCCTATGTCGCCTGCCTCG/GGGTGGACGAGGTCGCAGAA
fscRII RT-F/R	ATCGCATGTTGCTGGAGCGC/GGTGAGGAGGACGAGGGA
fscRIII RT-F/R	GAACACGAGGTGCGGTACG/TCGTCGGTGAGGAAGAGGC
fscRIV RT-F/R	GCCTGGTGCTCCTGGACGGT/CCACCCTGTACGGCTCCTG

[a]Underlining indicates restriction sites.

been removed from pApr-M by SpeI treatment, to generate pRII-L-A-R, thus connecting the left arm, the resistance cassette, and the right arm. The DNA segment with the two arms and apramycin cassette was released from pRII-L-A-R by HindIII and XbaI digestion, purified, and ligated with pJTU1278, pretreated with the same enzymes, to generate pMu-RII. The plasmid pMu-RII was transformed into *E. coli* ET12567(pUZ8002), and the resulting strain was used as the donor strain in conjugation with *Streptomyces* sp. FR-008. Exconjugants with resistance to apramycin were selected and verified by PCR using specific primers. One of these confirmed exconjugants was named the Δ*fscR2* strain. The same strategy was adapted to obtain the Δ*fscR3* strain. All primers used in this study are listed in Table 2.

For generation of strains with unmarked mutations, such as the Δ*fscR2* Δ*fscR3* strain, from which *fscR2* and *fscR3* were deleted, and the Δ*fscR1* Δ*fscR2* Δ*fscR3* Δ*fscR4* strain, from which all four regulatory genes were deleted, the two homologous arms were ligated directly without insertion of resistance genes and cloned into pJTU1278. Selection of exconjugants was performed as described previously (34).

Construction of complemented strains. To complement the *fscR2* mutation, the chromosomal region containing the 579-bp upstream and 152-bp downstream intergenic region and coding sequence of *fscR2* was amplified and cloned into the integrating plasmid pMS82 (52) to generate pCom-*fscR2* using a homologous recombination strategy; the construct was verified by sequencing and then introduced into the Δ*fscR2* strain to generate the complemented Δ*fscR2*-Com strain. Similarly, plasmid pCom-*fscR3*, containing the 319-bp upstream and 95-bp downstream intergenic regions and the coding sequence of *fscR3*, was used to generate the Δ*fscR3*-Com strain. Other complemented strains were obtained in a similar manner.

RNA isolation, RT, and real-time PCR. *Streptomyces* sp. FR-008 and its derivative strains were cultured in liquid YEME medium, and mycelia were collected at the time points indicated. Crude RNA samples were treated with DNase to remove chromosomal DNA. Reverse-transcription (RT) and real-time PCR assays were performed as described previously (8, 11, 55). Gene *hrdB*, encoding the major sigma factor, was used as an internal control to quantify the relative levels of cDNA.

RNA ligase-mediated RACE. *Streptomyces* sp. FR-008 total RNA was extracted and processed using the FirstChoice RLM-RACE kit (Ambion), as described previously (8). The 5′RACE outer primer (Ambion) and FscR3 (FscR2) outer primer were used for outer PCR. For inner PCR, the 5′RACE inner primer (Ambion) was paired with the FscR3 (FscR2) inner primer. PCR products were analyzed on an agarose gel, and specific bands were excised, purified, and cloned into pGEM-T-Easy (Promega). Multiple clones were sequenced to determine the transcriptional start point (TSP).

Bioassay and HPLC. The bioassay was performed essentially as described previously (34). Briefly, aliquots of supernatant collected during different growth phases in liquid YEME culture or patches of the agar medium were transferred to a thin layer of agar seeded with the indicator yeast strain *Rhodotorula rubra*, followed by incubation at 30°C for 48 h to test the growth inhibition of the indicator. Extraction and detection of the candicidin antibiotic complex from the wild-type *Streptomyces* sp. FR-008 were performed as described previously (26). The candicidin standard was purchased from Sigma, and the antimycin standard was purchased from MK Ltd.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.7 MB.

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