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Organizational and Mutational Analysis of a Complete FR-008/Candicidin Gene Cluster Encoding a Structurally Related Polyene Complex

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Summary

The complete gene cluster for biosynthesis of a polyene complex, FR-008, spans 137.2 kb of the genome of *Streptomyces* sp. FR-008 consisting of six genes for a modular PKS and 15 additional genes. The extensive similarity to the partially characterized candicidin gene cluster in *Streptomyces griseus* IMRU3570, especially for genes involved in mycosamine biosynthesis, prompted us to compare the compounds produced by *Streptomyces* sp. FR-008 and *Streptomyces griseus* IMRU3570, and we found that FR-008 and candicidin complex are identical. A model for biosynthesis of a set of four structurally related FR-008/candicidin compounds was proposed. Deletion of the putative regulatory genes abolished antibiotic production, while disruption of putative glycosyltransferase and GDP-ketosugar aminotransferase functionalities led to the productions of a set of nonmycosaminated aglycones and a novel polyene complex with attachment of altered sugar moiety, respectively.

Introduction

Polyene macrolides, such as amphotericin, nystatin, pimaricin, and candicidin, constitute a large class of natural products, many of which are effective clinical antifungal agents; some of them also have antiviral, antibacterial, or immunostimulating activities [1]. This group of polyketides, characterized by 3–8 conjugated double bonds in the macrolactone ring, usually form transmembrane channels by interacting with sterols in the eukaryotic cell membrane, causing leakage of small molecules and ions and consequent cell death [2].

Complete gene clusters for biosynthesis of amphotericin, nystatin, and pimaricin have been described, while the genes for candicidin were partially sequenced and analyzed [3–6]. The characteristic mode of biosynthesis of the polyketide backbone by modular PKSs, as first discovered for formation of 6-deoxyerythronolide B (6-dEB), the aglycone of the macrolide antibiotic erythromycin [7, 8] and later for many macrolide polyketide synthase (PKS) genes, such as those for avermectin [9], FK506 [10], rapamycin [11], rifamycin [12], and tylosin [13], is shared by polyene macrolides. In particular, each of the conjugated double bonds was found to be catalyzed by a specific PKS module. Thus, the modular organization typical of type I PKSs, consisting of repeated ketosynthase (KS), acyl transferase (AT), acyl carrier protein (ACP), and optionally, ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) domains, is clearly the general programming mechanism for biosynthesis of this important class of natural polyene compounds.

FR-008, a heptaene macrolide antibiotic with a 4-aminoacetophenone-containing aglycone identical to that of candicidin D, was reported to be produced by *Streptomyces* sp. FR-008, yet the exact chemical structure of the sugar moiety has not been determined [14, 15]. The isolated gene cluster, which was proven directly relevant to FR-008 biosynthesis [16], plus an additional cosmid for complete coverage of the biosynthetic pathway, was sequenced. Here, we describe an extensive organizational and mutational analysis of the FR-008/candicidin biosynthetic gene cluster by combined genetic, bioinformatic, and chemical approaches and generation of a series of novel FR-008/candicidin complex compounds by targeted gene disruptions or replacements.

Results and Discussion

The FR-008 Gene Cluster and Its PKS Genes

Complete sequencing of the inserts in five cosmids and one subclone from *Streptomyces* sp. FR-008 genomic library resulted in a continuous 137.2 kb DNA sequence. Computer-assisted analysis of the sequence revealed 21 potential ORFs putatively responsible for FR-008 biosynthesis as shown in Figure 1 and Table 1.

Six ORFs (*fscA-fscF*) encoding typical multifunctional type I PKS subunits, with exactly 21 PKS modules, were found, in agreement with the 21 condensation steps required for the synthesis of the carbon skeleton of FR-008 polyketide (Figure 1).

fscA is transcribed in the opposite direction to *fscC*, *fscB*, *fscF*, *fscE*, and *fscD*. The FscA protein seems to contain a loading module with an N-terminal putative ATP-dependent carboxylic acid-CoA ligase (CoL) and an ACP domain [6, 11, 17] for initiation of FR-008 aglycone biosynthesis, and module 1.

The FscB protein seemed to contain extension modules 2–4. All three AT domains in FscB have the consensus motif RVDVV×××××××M×S(A)×A××W preceding the active site GHS×G, thought to be characteristic of methylmalonate-specific AT domains (mAT) [18, 19] (Figure 2). DH4 in module 4 lacks the conserved se-

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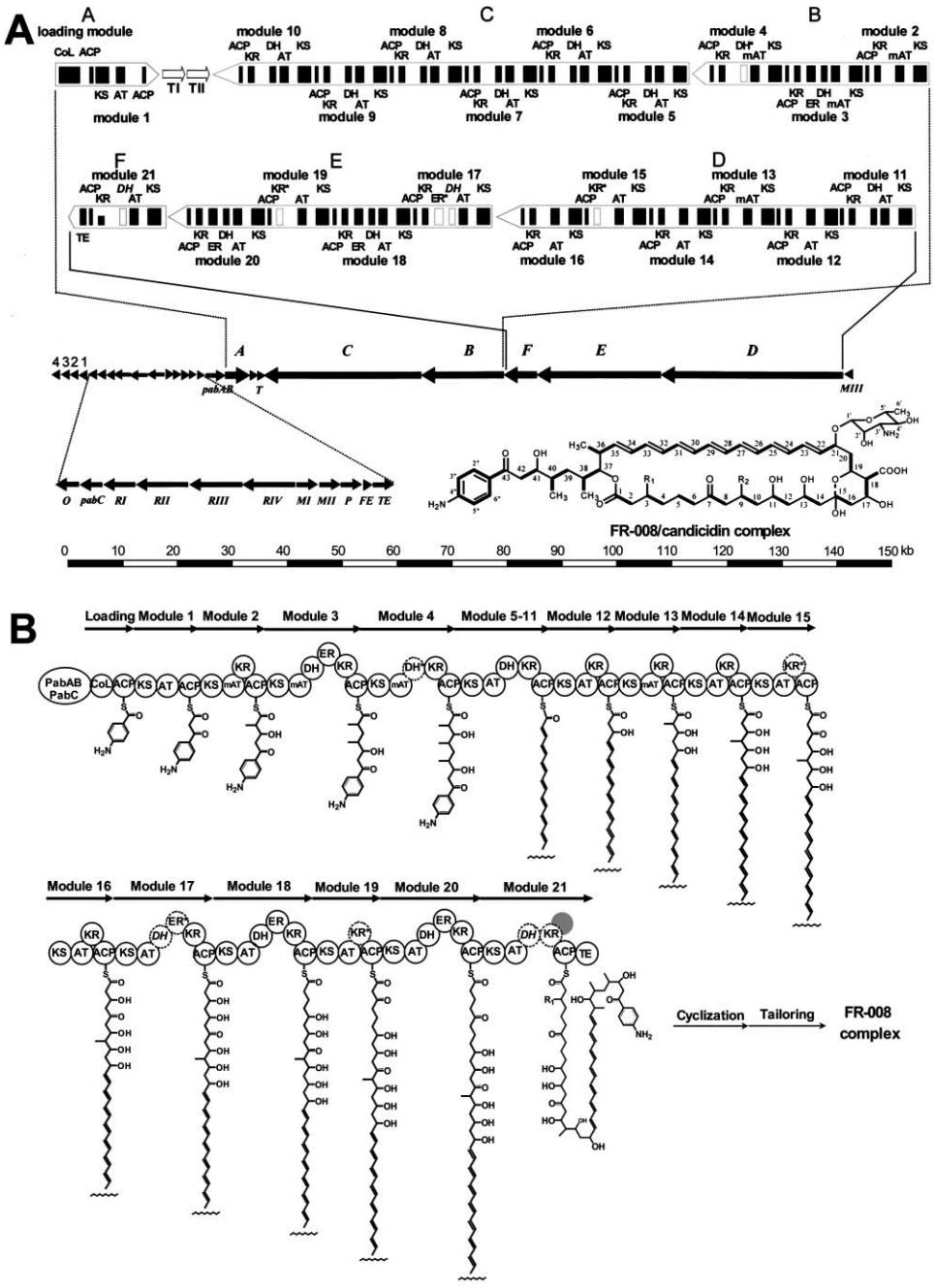

Figure 1. Organization of the FR-008 Gene Cluster and Proposed Model for Biosynthesis of the FR-008 Polyketide in *Streptomyces* sp. FR-008

(A) ORFs A–F represent the modular PKS genes, whose domain organization is detailed module by module. Active domains in ORFs are shown as solid black boxes while inactive (asterisks) or silent (italics) domains are shown as blank boxes. KR21 is shown by a smaller black box (details in the text).

(B) Each circle represents an enzymatic domain in the PKS with dashed circles representing the inactive or silent domains, and a dashed circle with back shadow represents KR21.

quence HPLL in the N terminus and is 56 aa shorter than the usual C terminus and therefore might be inactive (Figure 2).

FscC (10,625 aa) seemed to be a hexamodular protein, likely to be involved in the assembly of six (out of seven) conjugated double bonds, as its modules (5–10) all contain a DH-KR reduction loop.

FscD (9550 aa) is likely another hexamodular protein encoding modules responsible for elongation steps 11–16. Module 13 in FscD has a methylmalonate-specific AT domain (Figure 2). Modules 12, 14, 15, and 16 all have the same KS-AT-KR-ACP domain structure, but KR15 lacks the first two conserved G residues within the NADP binding motif G×G××G×××A (Figure 2) and

Table 1. Deduced Functions of the ORFs Identified in the FR-008 Gene Cluster and Comparison with Other Polyene Pathway Genes

FR-008			Homologous Genes in Other Polyene Pathways			
Gene/aa No.	Product	Deduced Function	Nys	Amph	Pim	Can
pabAB/723	ADC synthase	biosynthesis of starter unit PABA	–	–	–	*pabAB*
pabC/257	ADC lyase	biosynthesis of starter unit PABA	–	*orf2*	–	–
fscO/458	FAD-dependent monooxygenase	putative tailoring enzyme	–	–	–	–
fscA/1,743	type I PKS	loading module and module 1	*nysA*	*amphA*	*pimS0*	*canP1*
fscB/5541	type I PKS	modules 2–4	*nysB*	*amphB*	*pimS1*	*canP2*
fscC/10,625	type I PKS	modules 5–10	*nysC*	*amphC*	*pimS2*	*canP3*(P)
fscD/9550	type I PKS	modules 11–16	*nysI*	*amphI*	*pimS3*	*canPF*(P)
fscE/7771	type I PKS	modules 17–20	*nysJ*	*amphJ*	*pimS4*	–
fscF/2049	type I PKS	modules 21 + TE	*nysK*	*amphK*		–
fscRI/222	transcriptional regulator	regulation	*orf4*	–	–	–
fscRII/942	transcriptional regulator	regulation	*nysRIII*	–	–	*orf1*(P)
fscRIII/1036	transcriptional regulator	regulation	*nysRII*	–	–	*orf2*
fscRIV/1005	transcriptional regulator	regulation	*nysRI*	–	–	*orf3*
fscMI/458	glycosyltransferase	attachment of mycosamine	*nysDI*	*amphDI*	*pimK*	*canG*
fscMII/352	GDP-ketosugar aminotransferase	mycosamine biosynthesis	*nysDII*	*amphDII*	*pimC*	*canA*
fscMIII/402	GDP-mannose-4, 6-dehydratase	mycosamine biosynthesis	*nysDIII*	*amphDIII*	*pimJ*	*canM*(P)
fscP/393	cytochrome P450 monooxygenase	formation of carboxyl group at C-18	*nysN*	*amphN*	*pimG*	*canC*
fscFE/64	ferredoxin	electron transfer in P450 system	*nysM*	*amphM*	*pimF*	*canF*
fscTE/285	type II thioesterase	removal of aberrant intermediates	*nysE*	–	*pimI*	*canT*
fscTI/335	ABC transporter	efflux of FR-008	*nysG*	*amphG*	*pimA*	*canRA*
fscTII/239	ABC transporter	efflux of FR-008	*nysH*	*amphH*	*pimB*	*canRB*

could thus be inactive so as to leave C-15 unreduced as a carbonyl group.

FscE might be a tetramodular protein responsible for elongation steps 17–20. The ER domain in module 17 (KS-AT-DH-ER-KR-ACP) is 67 aa shorter than ER3, ER18, and ER20 in the N terminus (Figure 2) and is probably inactive. Recently, KR domains of modular PKSs and animal FASs were shown to comprise a unique subfamily of reductases within the SDR superfamily of enzymes, with conserved Tyr, Ser, and Lys residues as a catalytic triad in the active site, and a mutated Tyr in the DEBS pathway of the *ery* cluster was found to eliminate KR6 activity completely [20]. KR19 shows a Tyr to Leu replacement and would thus seem to be inactive (Figure 2). In a similar way, the inactive KR15 domain in FscD (see above) also shows a Tyr to Gly replacement in the active site (Figure 2).

A chain-terminating thioesterase (TE) domain is presumably present at the C-terminal end of FscF, preceded by module 21. The DH domain in FscF seems to be intact, but its activity would not be required at the last condensation step (an identical situation was found for DH17 of FscE). This is reminiscent of similar situations, in which intact but nonfunctional DH domains were observed in the last module (NysK) of nystatin [4] and two internal modules (RifB and RifC) of the rifamycin [12] and amphotericin PKS (AmphJ) [3]. Compared with known KR domains, KR21 seems to be an intact domain with no detectable differences in any of the active motifs and residues, but structural analysis of the FR-008 complex suggests its action, as will be discussed later.

The TE domain would enable FscF to release the mature FR-008 polyketide chain from the PKS to form a lactone. Besides the type I TE domain in FscF, a gene (*fscTE*), putatively encoding a type II thioesterase, was found immediately upstream of *pabAB* (Figure 1). The behavior of the tylosin TEII, which was proposed to remove unreacted acetyl, propionyl, or butyryl groups on the ACP thiol from the multienzyme complex based on its action on SNAC thioesters in vitro [21], suggests it to be an editing enzyme, removing aberrant intermediates that might block further extension of the chain and so maintaining normal levels of antibiotic production [22, 23]. The work on the pikromycin TEII (PicAV) showing unequivocal acyl-ACP hydrolysis with the physiological substrates in vitro [24] tends to further support the editing role of TEII.

PABA Synthase Genes

p-aminobenzoic acid (PABA) is expected to be the precursor of the aromatic *p*-aminoacetophenone moiety that would serve as the starter unit for FR-008 polyketide synthesis. PabAB (723 aa), a likely heterodimer protein sandwiched between *fscTE* and *fscA* (Figure 1), encodes a fused 4-amino-4-deoxychorismate (ADC) synthase with 93% identity to PabAB from *S. griseus* IMRU3570 and 46% identity to PabAB from *S. venezuelae*. Both glutamine amidotransferase (class I) and chorismate binding motifs were detected in PabAB, suggesting its proposed functions as glutaminase and chorismate aminase (a likely function of PabB is to convert chorismate and glutamine to 4-amino-4-deoxychorismate [ADC] and glutamate, supported by PabA), as in *Escherichia coli* [25, 26].

pabC, lying immediately upstream of the putative regulatory gene *fscRI* (Figure 1), encodes a 257 aa protein that resembles 4-amino-4-deoxychorismate (ADC) lyases [27] from numerous bacteria, including *Mycobacterium tuberculosis* H37Rv (32% similarity and 24% identity) and *Pseudomonas aeruginosa* PA01 (32% similarity and 23% identity). PabC most probably fulfills its function by converting ADC generated by PabAB to the aromatized PABA and pyruvate and is thought to be a kind of aminotransferase or closely related pyridoxal-5′-phosphate-dependent enzyme [27]. A PabC homolog has not been discovered in the intensively studied

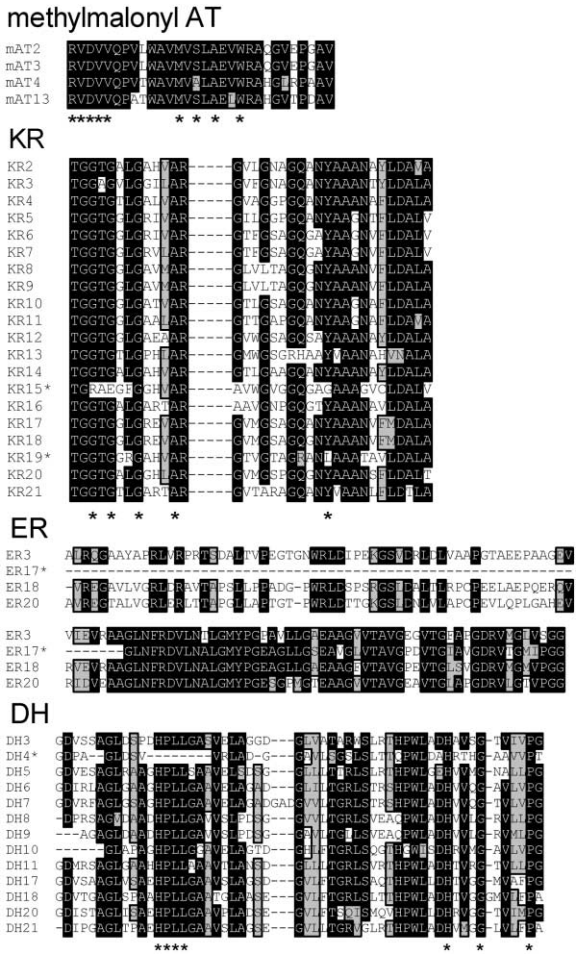

Figure 2. Alignment of Active Site Sequences of Grouped Domains from FR-008 PKSs

Only the regions containing the proposed active sites are shown. Active site residues are marked with asterisks.

strains *S. griseus* IMRU3570 (candicidin producer) and *S. venezuelae*. Interestingly, PabC in FR-008 is 33% identical with AmphORF2, although it is predicted that *pab* genes would not be required for amphotericin biosynthesis because of the absence of an aromatic moiety.

Modification and Transport Genes

FscP and FscFE would represent a P450 monooxygenase system, with functions similar to AmphN, NysN, and PimG, likely responsible for oxidation of the C-18 methyl branch to a carboxyl group, which is presumably introduced in the 13th elongation step by the methylmalonate-specific AT13 of FscD. Almost all of the P450 monooxygenases acting on PKS structures stop at the alcohol, thus some additional enzyme, such as an oxidoreductase (conversion of the primary alcohol to the aldehyde), would seem to be required in the process of converting a methyl branch to a carboxyl group, although no such gene(s) had been identified in all of the completely characterized polyene gene clusters [3–5] and such function(s) might be recruited from elsewhere in cellular metabolism.

FscO, a FAD-dependent monooxygenase, showing 34% similarity and 25% identity to a FAD-dependent monooxygenase from *Agrobacterium tumefaciens* C58, was found to lie immediately downstream of PabC at the left end of the gene cluster (Figure 1). FscO most probably acts as a tailoring enzyme whose role will be discussed later.

Between the two PKS genes *fscA* and *fscC* lie two genes (*fscTI* and *fscTII*) whose deduced products resemble proteins belonging to the ATP-dependent ABC transporter superfamily from *S. coelicolor* A3(2), which are probably involved in efflux of the FR-008 antibiotic.

Putative Regulatory Genes

Four putative regulatory genes (*fscRI*, *fscRII*, *fscRIII*, and *fscRIV*), whose deduced products all belong to the LuxR family of transcriptional regulators, might be involved in regulation of FR-008 biosynthesis. Such transcriptional regulators may act to maintain the stability of the extremely long mRNAs of the large PKS genes.

The putative regulatory activities of the above genes for FR-008 biosynthesis were further supported when the 6414 bp DNA (3811–10,225 of the GenBank accession number AY310323) carrying two of the four clustered genes, *fscRII* and *fscRIII*, and a partial *fscRI* (but not *fscRIV*), were replaced by a cassette carrying the apramycin [*aac3*(*IV*)] and erythromycin (*ermE*) resistance genes (see Experimental Procedures). The engineered mutant (HJ1) no longer produced any FR-008-related compounds, as determined by bioassay using *Saccharomyces cerevisiae* Y029 as an indicator strain as well as by HPLC analysis (data not shown).

The Boundaries of the FR-008 Gene Cluster

Hu et al. [16] engineered mutations by targeted gene disruptions, using the three leftmost BamHI fragments from pHZ145 to mediate homologous recombination, yielding three mutants that were not affected in FR-008 production, suggesting that these DNA fragments are outside the biosynthetic gene cluster. The DNA sequence of one of the three fragments lies immediately to the left of *orf1-orf4* (Figure 1), which seem to encode four branched-chain amino acid ABC transporters involved in amino acid metabolism. Only two transporter genes (PimA and PimB for pimaricin, NysG and NysH for nystatin, and AmphG and AmphH for amphotericin) [3–5], equivalent to *fscTI* and *fscTII* for FR-008, seem to be necessary as efflux proteins in all of the completely characterized polyene gene clusters. The leftmost *orf1-orf4* (putative additional ABC transporters) were assumed no role in efflux of FR-008, but whether the exact left boundary of the gene cluster would exclude *orf1-orf4* remains to be experimentally determined.

To the right of *fscMIII* is an incomplete ORF whose deduced product resembles the hypothetical protein SC5F.14c and the putative secreted protein SCE34.21c (SCO3040) from *S. coelicolor* A3(2), both of which harbor a conserved PGRP domain (animal peptidoglycan recognition protein) homologous to bacteriophage T3 lysozyme. No such function(s) could be assigned in FR-008 biosynthesis and, in addition, no resemblances to any genes in the nystatin, amphotericin, candicidin, or pi-

maricin clusters could be detected. These data suggested that this incomplete gene is the right boundary of the FR-008 cluster.

Putative Genes for Biosynthesis of an Undetermined Sugar and Its Attachment to the FR-008 Aglycone

Three genes were identified for biosynthesis of the previously undetermined sugar moiety and its attachment to FR-008 aglycone. FscMII (352 aa, Figure 1) was 92% identical to CanA in the candicidin pathway and 74%–76% identical to AmphDII, NysDII, and PimC, all of which seemed to encode putative GDP-ketosugar aminotransferase activities involved in mycosamine biosynthesis. *fscMIII* encodes a 402 aa protein with 74%–76% similarity and 66%–69% identity to GDP-mannose-4,6-dehydratases (PimJ, NysDIII, and AmphDIII). Therefore, FscMIII and FscMII seem to be involved in biosynthesis of mycosamine (3,6-dideoxy-3-amino-D-mannose).

To the left of FscMII is FscMI (458 aa, Figure 1), whose aa sequence resembles UDP-glucuronosyltransferase (AmphDI, NysDI, and PimK) over their entire length. It is likely to be responsible for attaching a sugar to the aglycone of FR-008 at C-21.

The above comparative analysis of the roles of FscMIII, FscMII, and FscMI implys that the previously undetermined sugar moiety in FR-008 might be mycosamine, as the genes for its biosynthesis and attachment to different aglycones in all identified polyene systems seemed highly homologous. This assumption was confirmed by reexamination of the chemical structures of the FR-008 molecules by LC-MS_1/MS_2 and NMR and by the chemical analysis of the derivative compounds in *fscMI* and *fscMII* gene disruptants (see below).

FR-008 and Candicidin Gene Clusters Are Highly Homologous, and FR-008 and Candicidin Complex Are Identical

The organization and characteristics of the complete FR-008 gene cluster deduced from the 137.2 kb DNA sequence was compared with DNA (39,314 bp and 1020 bp) and deduced protein sequences available in the public database for candicidin biosynthesis [6]. The homologous genes available for comparison are similar in size and identical in order and direction of transcription, and the nucleotide bases between 5421 and 44,805 of the FR-008 gene cluster (GenBank accession number AY310323) are c. 97% identical to the 39,314 bp sequence from the candicidin gene cluster (GenBank accession number AJ300302).

Only two of the candicidin PKS genes (*canP1* and *canP3*) are sequenced completely and another (*canP2*) is partially sequenced. The counterpart FscA protein of the FR-008 gene cluster is c. 97% identical to CanP1.

Among other sequenced counterpart genes, noticeable resemblances were observed for a small protein FscFE (64 aa; 100% identical to CanF), for FscP (96% identity with CanC), for PabAB (93% identity with PabAB from candicidin), and for FscTI and FscTII (85% and 76% identity with CanRA and CanRB, respectively). Another part of a sequenced region (1020 bp) (GenBank accession number AJ300303) in the candicidin gene cluster showed high identity to a corresponding region in the FR-008 cluster, including FscD (1–201 of the 1020 bp sequence encoding an obvious KS domain) and a partial FscMIII (631–1020 of the 1020 bp sequence). Such a high degree of sequence identity at both nucleotide and amino acid level between the two pathways suggests a common ancestry of the two gene clusters and that the chemical structures of the FR-008 and candicidin antibiotic complex might be identical.

HPLC separation profiles of antibiotic compounds produced by *Streptomyces* sp. FR-008 and the candicidin complex produced by *S. griseus* IMRU3570 were largely indistinguishable (Figure 3). When the compounds isolated from the two strains were mixed, the retention time (RT) of each of the four major peaks expected for the FR-008/candicidin complex was unchanged (Figure 3), and the UV spectra of FR-008-I, II, III, and IV were exactly the same as for the candicidin complex (data not shown). When each peak was subjected to LC-MS_1/MS_2 analysis in negative mode, almost identical data were obtained for each peak from both samples and for the mixture (Figure 3). It is therefore likely that *Streptomyces* sp. FR-008 produces four related compounds identical to the candicidin complex produced by *S. griseus* IMRU3570.

A Proposed Model for the Generation of the Four Structurally Related FR-008 Compounds

FR-008-II (Figure 3) seemed to be an isomer of FR-008-III (Figure 3) as its *m/z* 1108 is identical to that of FR-008-III and their counterpart peaks corresponded to candicidin-II and -III (*m/z* 1108, Figure 3), whose theoretical molecular weight (1109) is the same as that of candicidin D ($C_{59}H_{84}N_2O_{18}$, MW 1109) [28]. Detection of chemical shifts of the protons related to the determination of some major functional groups in FR-008-II by analysis of the ^1H-^1H COSY spectrum [29], together with data from ^{13}C NMR analysis of FR-008-II (Figure 4) support its structure, as shown in Figure 4. Obviously, no hemiketal ring between C-15 and C-19 in FR-008-II was detected, and one ketal at δ 96.9 is characteristic of C-1' of the mycosamine, and four ketones between δ 202.2 and δ 196.7 are characteristic of C-3, C-7, C-15, and C-43, respectively. The six-membered hemiketal ring is likely to be due to the detected isomer FR-008-III equilibrated in the FR-008 complex, as was similarly reported by Omura and Tanaka [30]. Structural analysis of amphotericin B, a polyhydroxy ketone, which was suggested to have structural features adequate for the formation of equilibrium amounts of various cyclic ketal forms, proved by coexistence of a six-membered ring cyclic ketal structure with the non-six-membered ketalic ring structure [31], tends to support the above conclusion.

The difference of mass between FR-008-I and FR-008-II (III) is 2 Dalton, which could be explained if KR21 activity is not efficient enough to catalyze complete keto reduction prior to passage of the polyketide chain intermediate onto the TE, or alternatively, the TE could not distinguish sufficiently between the oxidized form of the polyketide and the form that had been reduced by KR21 prior to cyclization, leading to a compound mixture with keto and hydroxyl groups at the C-3 position (Figure 4).

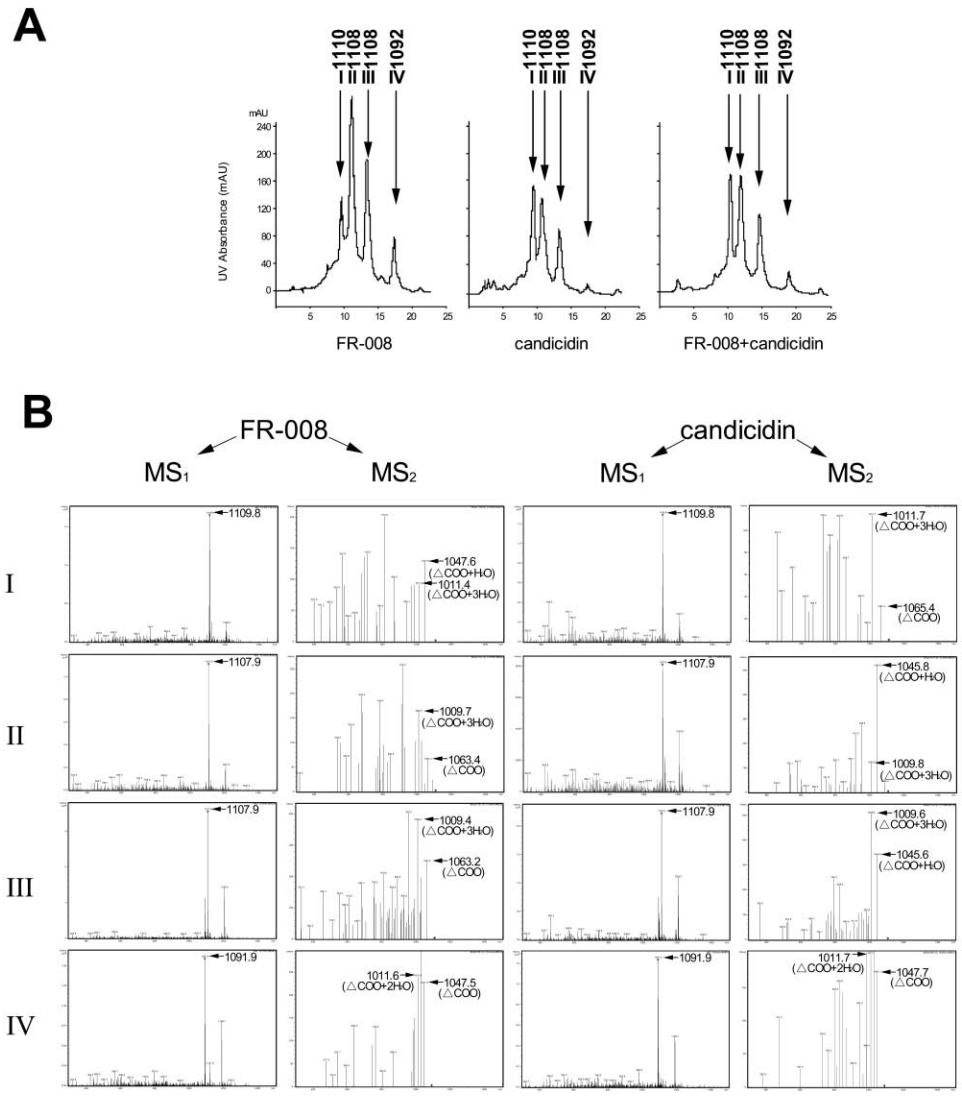

Figure 3. Comparison of FR-008 and Candicidin Complex

(A) HPLC analysis of antibiotic FR-008, candicidin, and their mixture, whose peaks and respective retention times seem to be identical. Their corresponding MS_1 (m/z) data are indicated by arrows above the HPLC peaks.

(B) The LC-MS_1/MS_2 fragmentation (loss of H_2O or COO, ΔH_2O, or ΔCOO) patterns of FR-008/candicidin I–IV, respectively.

The MS_1 *m/z* 1092 of the FR-008-IV/candicidin-IV has a MW difference of 16 Dalton from FR-008-II (III)/candicidin-II (III) (MS_1 *m/z* 1108). The correlation of the possible structural features between FR-008-IV/candicidin-IV and FR-008-II (III)/candicidin-II (III) with putative FscO (FAD-dependent monooxygenase) function poses several alternative possibilities (only the first possibility is indicated in the relevant part of the figures). First, it could be that a hydroxyl group that was initially removed by functional DH-ER in module 18 during polyketide biosynthesis was restored at C-9 after polyketide formation (Figure 4), but this mechanism, involving a proposed C-9 hydroxylation, which would occur at a position derived from C1 (instead of C2) of an acetate unit, was unprecedented and thus questionable. An alternative possibility might be that FscO hydroxylates FR-008/candicidin aglycone at C-10 (C2 of an acetate unit) similar to hydroxylations at C-10 and C-8 of the nystatin and amphotericin aglycones by NysL and AmphL, respectively, but such a suspicion for FR-008 and/or candicidin structures could not be clearly distinguished by mass spectrometric analysis either in the present study or in Zielinski et al. [28]. Third, if DH18 were assumed silent as DH21 and DH17, a hydroxyl group would be left unreducted at C-9. FscO could then be anticipated as a role for conversion of the C-18 hydroxyl to an aldehyde, resulting in a structure of an intermediate with the same MS_1 (1092). However, fragmentation pattern (MS_2/MS_3) of FR-008-IV outlined in Figure 3, which indicates a clear loss of a carboxyl (−COO) moiety, seems to disagree with this possibility.

Thus, the above hypothesis for the coproduction of several FR-008/candicidin compounds in both strains, largely based on the analysis of the complete gene clus-

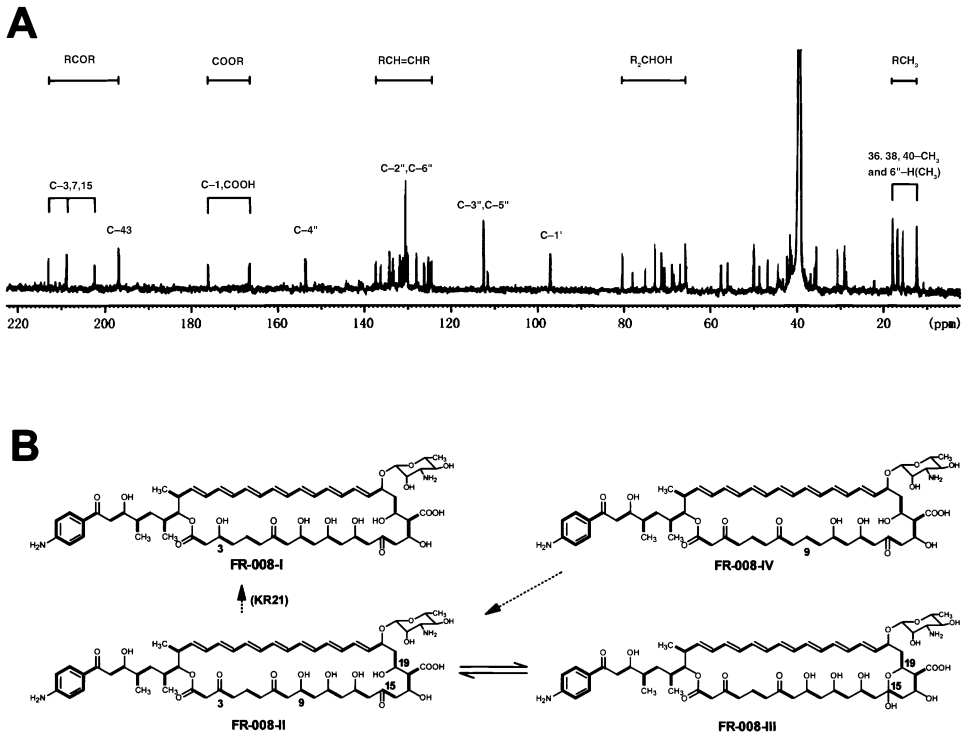

Figure 4. ^{13}C NMR Data for FR-008-II in DMSO and Hypothetical Mechanism for Structural Variations

(A) ^{13}C NMR data for FR-008-II in DMSO.

(B) Hypothetical mechanism for structural variations: incomplete keto reduction of FR-008-II catalyzed by KR21 (indicated as a dashed arrow) at C-3 position before subsequent cyclization is assumed to be the result of coexisted FR-008-I. Another dashed arrow points to an uncertain tailoring step between FR-008-IV and FR-008-II (see discussion in the text).

ter for antibiotic FR-008, will have to be tested by ongoing experiments to activate/inactivate specific gene(s) (e.g., *fscO* and/or *fscP*) or domain (KR21) and look for the expected structural changes.

Removal of Mycosamine from All Four Closely Related FR-008/Candicidin Compounds by Targeted Disruption of the *fscMI* Gene

The putative FscMI protein (Figure 1) is assumed to catalyze attachment of mycosamine to C-21 of the four aglycones (Figure 1) in the FR-008/candicidin complex. Thus, disruption of this gene should produce the corresponding aglycones (Figure 5). An engineered mutant (CS101 in Figure 5) with *fscMI* interrupted by *aac(3)IV* (apramycin resistance gene) was created (as described in Experimental Procedures and outlined in Figure 5). When five independent CS101 isolates were tested for activity against *Saccharomyces cerevisiae* Y029, turbid inhibition zone could be detected, in sharp contrast with the clear inhibition observed with stain FR-008, which indicates at least a reduced potency. The expected structural changes in all four derivative compounds were confirmed. LC-MS_n analysis of the extracts of the CS101 cultures revealed no *m/z* data corresponding to the theoretical masses, 1110 for FR-008-I, 1108 for FR-008-II and FR-008-III, and 1092 for FR-008-IV in MS_1, but MS_1 *m/z* data (965, 963, 963, and 947) for four major peaks showing characteristic absorption spectra of heptaene macrolide [30] were seen corresponding to the theoretical masses of the individual aglycones of FR-008-I, FR-008-II, FR-008-III, and FR-008-IV, respectively. In all four compounds there was a mass difference corresponding to substitution of a hydrogen (MW 1) for the mycosamine moiety (MW 146). The chemical structures of individual aglycones were further confirmed by their major fragmentation patterns detected in MS_2 and/or MS_3 (Figure 5).

Interestingly, a new HPLC peak, characteristic of absorption spectra of heptaene macrolides (Figure 5) with a *m/z* 933, corresponding to the theoretical mass of a noncarboxylated and nonmycosaminated derivative (MW 934) of FR-008-II and FR-008-III, appeared and was further confirmed by LC-MS_1/MS_2/MS_3 (Figure 5). Probably the lack of glycosylation caused feedback/product inhibition of other post-PKS tailoring steps, such as the hydroxylation of the C-18 methyl group.

Generation of Novel FR-008 Derivatives with/without Attachment of Mycosamine Precursor by Disruption of *fscMII* Gene

FscMII was assumed to be responsible for transamination of GDP-3-keto-6-deoxy-D-mannose to form GDP-mycosamine, and it was thus anticipated that disruption of *fscMII* would result in attachment of an altered sugar moiety (3-keto-6-deoxy-D-mannose) to the corresponding aglycones, if the altered substrates could still be recognized by the glycosyltransferase (FscMI).

To prove the role of FscMII as a transaminase in myco-

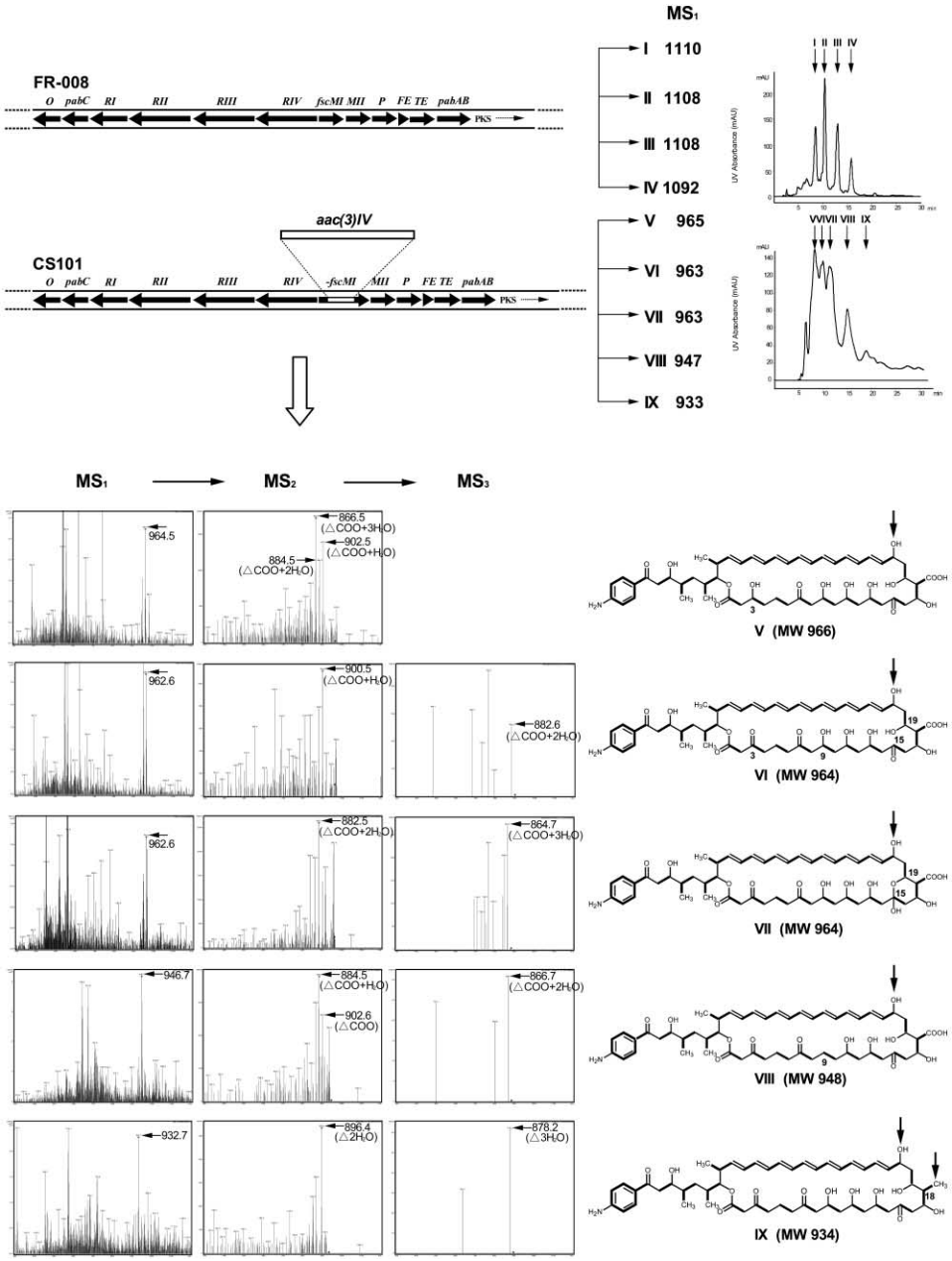

Figure 5. Generation of FR-008 Derivative Compounds by Insertion of an Apramycin Resistance Gene in *fscMI* in the Wild-Type *Streptomyces* sp. FR-008

The upper part shows the region flanking the *fscMI* gene in the chromosome of the wild-type strain of *Streptomyces* sp. FR-008, beside which are listed MS_1 (m/z) data corresponding to four HPLC peaks (I–IV). The middle part shows a mutant, CS101, created by targeted disruption of a specific *fscMI* region (indicated as −*fscMI*) with *aac(3)IV* (a horizontal bar above CS101), beside which are listed MS_1 (m/z) data corresponding to five HPLC peaks (V–IX) of derivative compounds, whose chemical structures (right) deduced from LC-MS_n analysis (left) were all shown to have presumably lost mycosamine moieties. Only numbered LC-MS_1/MS_2/MS_3 fragmentation data for each successive step relevant to each of indicated structures were amplified to show *m/z* changes after loss of specific groups [ΔCOO and/or $\Delta(H_2O)_n$].

samine biosynthesis and to generate FR-008 derivatives with attachment of deaminated sugar moiety, an engineered mutant (CS102 in Figure 6) with disruption of *fscMII* by *aac(3)IV* (apramycin resistance gene) was obtained (as described in Experimental Procedures and indicated in Figure 6). To our surprise, when fermentation products from CS102 were subjected to LC-MS_1, FR-008 aglycones without sugar moiety (MS_1 *m/z* data 965, 963, and 947), which were exactly the same as compounds V–VIII from CS101 (Figure 5), were found to be the predominant products, although three novel compounds, whose MS_1 *m/z* data (1109 for X, 1107 for XI, and 1091 for XII) corresponds to the sum of the respective aglycones and 3-keto-6-deoxy-D-mannose (MW 1110, 1108, and 1092), were also detected at lower level. The approximate ratio between aglycones and the

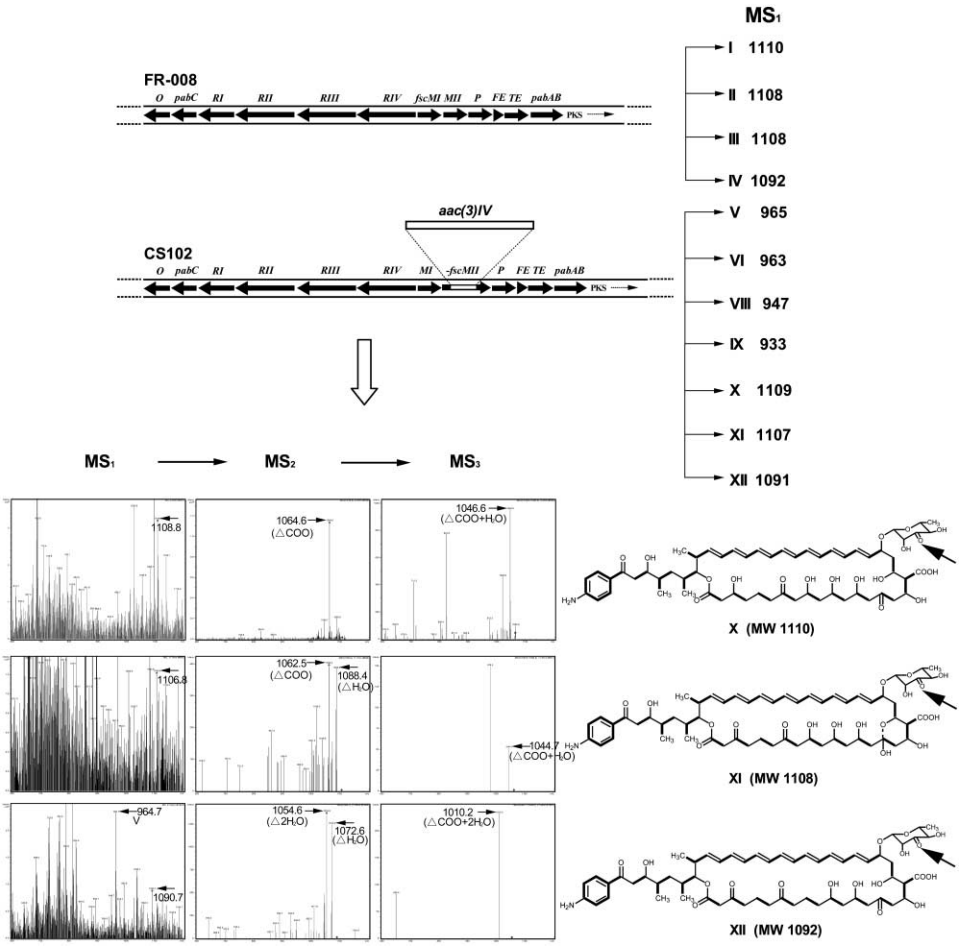

Figure 6. Creation of Novel FR-008 Derivative Compounds by *fscMII* Gene Disruption

Novel FR-008 derivative compounds with attachment of a different sugar moiety (3-keto-6-deoxy-D-mannose) other than mycosamine (lower part) by insertion of an apramycin resistance gene [*aac(3)IV*] in *fscMII* in the wild-type *Streptomyces* sp. FR-008 (upper part) are shown, as in Figure 5.

novel compounds (X–XII in Figure 6) containing the 3-keto-6-deoxy-D-mannose ranges 5–10:1, and the amount of the aglycones produced is about the half of the normal FR-008/candicidin complex. The production of a non-carboxylated and nonmycosaminated FR-008-II derivative (MW 934, IX in Figure 5) was unaffected. All of the above mentioned compounds produced by CS102 were confirmed by LC-MS_n fragmentations, but only fragmentation patterns of the three novel compounds with an altered sugar moiety (X, XI, and XII) are shown in Figure 6.

GDP-mycosamine differs from its precursor, GDP-3-keto-6-deoxy-D-mannose, only with a change of an amino group to a keto group at C-3, but it is obvious that the flexibility of FscMI (glycosyltransferase) to recognize the two different substrates seemed to be limited. Again, reduced inhibition of CS102 to *Saccharomyces cerevisiae* Y029, as were similarly observed for CS101 when compared in parallel with the clear inhibition displayed by the wild-type FR-008/candicidin compounds, was seen. The antifungal activities of the FR-008/candicidin derivatives with deaminated sugar moiety in a mixture containing the nonglycosylated aglycone structures were thus not determined unambiguously.

Advantages of Using *Streptomyces* sp. FR-008 for Handling Polyene Genes

Establishment of reliable gene transfer systems allowing efficient genetic manipulation of the polyene-producing *Streptomyces* experienced great difficulty in several polyene producers, including *S. griseus* IMRU3570 (producing candicidin). So far, only interspecific conjugation and phage-mediated gene transfer allowed the efficient introduction of recombinant DNA into these strains [3, 6, 17, 32]. Our systems for handling *Streptomyces* sp. FR-008, however, are robust and amenable, including successful construction of the restriction-deficient mutant host strain DX600 [16] allowing highly efficient introduction of the autonomously replicating cosmid pHZ132 [33], pIJ653, *E. coli* suicide vector pHZ199, and pDH5 by transformation or conjugation, targeted gene knockouts using unstable low (pKC505 derived from SCP2*) [16] and high copy number (pHZ1358 derived from pIJ101) [34, 35] *Streptomyces* plasmid vectors. Added to the advantage of *Streptomyces* sp. FR-008 is its exceptionally fast growth rate and very abundant sporulation. The successful disruptions of *fscMI*, *fscMII*, and some regulatory genes using pHZ1358 described in the present

study constituted the most recent and convenient examples.

Significance

Although several gene clusters for biosynthesis of polyene macrolide antibiotics have been cloned and sequenced, the one for the well-known candicidin has been incomplete. The extensive resemblances of the genes between *Streptomyces* sp. FR-008 and the partially reported candicidin pathway in *S. griseus* IMRU3570 led to a chemical comparison between four structurally related members of a FR-008/candicidin antibiotic complex produced by both organisms and the proposal of a genetic structure relationship for the possible interconversion of the members of the FR-008/candicidin complex, as well as a complete picture for overall FR-008/candicidin biosynthesis. A role for PabC, a protein involved in the conversion of 4-amino-4-deoxychorismic acid (synthesized by ADC synthase, PabAB) to PABA, as in *E. coli*, seems to be the first reported in *Streptomyces*. Targeted disruption of the specific genes generated a series of compounds derived from the FR-008/candicidin complex with loss or altered sugar moieties, confirming the role of FscMI as a relatively flexible glycosyltransferase and FscMII as transaminase by genetic approaches. Conceivably, this observation would open more possibilities for the flexible tailoring by attachment of the altered sugars to the FR-008/candicidin aglycones to create great diversity of compounds. The efficient genetic manipulation of FR-008 gene cluster demonstrated clearly that *Streptomyces* sp. FR-008 could serve as a model strain for either fundamental or applied researches aiming at generation or interconversion of novel polyenes or related compounds using the FR-008/candicidin gene clusters, or in combination with genes from other pathways.

Experimental Procedures

Bacterial Strains and Plasmids

Streptomyces sp. FR-008, the wild-type producer of the antibiotic FR-008, and *S. griseus* IMRU3570, the producer of candicidin (a kind gift of Dr. José Gil), were used for the isolation of antibiotics and bioassay.

DH5α (F^-, *rec*A, *lac*Z, ΔM15) [36] was used as *E. coli* host. pHZ132 [33] is a cosmid for constructing the FR-008 genomic library, pHZ1358 [34, 35] is a vector for constructing plasmids for targeted gene disruption or replacement, and pBluescript II SK(+) [37] was used as vector for constructing pJTU6.

General Techniques

Recombinant DNA techniques were as described by Sambrook et al. [38]. PCR reactions were performed using High Fidelity PCR Master (Roche).

We labeled the probe with ^{32}P-dCTP by using a Nick Translation Kit (Roche). Hybridization was done in Hybridizer HB-1D (Techne), and signals were detected with a phosphoimager (Fujifilm FLA3000). Hybridization using probes labeled with digoxigenin-labeled dUMP (Roche) was carried out as specified by the manufacturer at 68°C.

Antibiotics, Preparation, Bioassay, and Structural Characterization

Candicidin sample was a kind gift from Dr. José Gil. For NMR, 10 liters of the culture broth of *Streptomyces* sp. FR-008 were extracted with 10 liters of n-butanol. After removal of n-butanol in vacuum, the residue was dissolved in 60% methanol and applied to a column of macroreticular neutral resin XAD-16 (Sigma). After washing the column with two volumes of 50% methanol, the active fraction was eluted with 80% acetone and concentrated in vacuum to obtain ca. 800 mg of brown powder. The powder was then applied to a silica gel column charged with 80% acetone and eluted with the same solvent. After concentrating and drying, 100 mg of a yellow powder of FR-008 complex was obtained.

The antibiotics in five plates of individual CS101 and CS102 were extracted for antibiotic bioassay with *Saccharomyces cerevisiae* Y029 as an indicator.

Silica gel column was omitted for extraction of 200 ml culture broth for the LC-MS_n analysis, using the Agilent 1100 series LC/MSD Trap system. The LC was operated at a flow rate of 0.6 ml min^{-1} with a Waters XTerra™ RP18 (3.9 × 150 mm, 5 μm) column. The eluent was 38% acetonitrile and 62% 0.05 M NaH_2PO_4/Na_2HPO_4 (pH 6.0). The iontrap mass spectrometer was operated with the electrospray ionization source in the negative ion mode. Drying gas flow was 10 l/min, and nebulizer pressure was 50 psi. Drying gas temperature was 325°C. The fragmentation amplitude was varied between 1.0 to 1.8 V.

NMR spectra were measured with a Bruker AM 500 NMR spectrometer in DMSO-d6 at room temperature at 125 MHz (^{13}C), using the solvent as internal reference downfield of TMS at 0 ppm. ^{13}C NMR was recorded using broadband proton decoupling.

DNA Sequencing

DNA sequencing was performed at Genotech Ltd. (Taejon, Korea) using a set of five cosmids (pHZ145, pHZ137, pHZ220, pHZ194 [16], and pJTU1) and a subclone (in pJTU6) linking cloned BamHI fragments from cosmids pHZ137 and pHZ220 [16]. The rightmost non-PKS region of pHZ194 [16] was found to be a recombinant DNA fragment obtained during library construction and is thus unrelated to FR-008 production. pJTU1 is one of the two cosmids (pJTU1-2) flanking and overlapping with the right-hand end of the PKS region of pHZ194 obtained by Southern hybridization against a cosmid library constructed in pHZ132 [33] by using as probe the short unconserved region between the sequenced PKS domains in pHZ194. Two primers used for obtaining a 218 bp DNA probe for screening pJTU1-2 with *Streptomyces* sp. FR-008 genomic DNA as template are CS1 (5′-TGCCGCGCTCGCCGACA-3′) and CS2 (5′-CGCGTCCG GTGCTCACG-3′).

The sequence data were analyzed with the Frame-Plot 2.3 online program [39]. DNA and deduced protein sequence homology searches were performed using BLAST [40–42] and FASTA [43]. Multiple alignments of sequences were done using CLUSTAL W [44].

Generation of Insertions in *fscMI* and *fscMII* by Targeted Gene Disruption

A 6.6 kb KpnI fragment isolated from pHZ145 was cloned into the corresponding site of pIJ2925 to give pJTU26. The 1.4 kb EcoRV-SmaI blunt-end fragment from pHGF9827 (L. Bai, personal communication) carrying the apramycin resistance gene [*acc(3)IV*] was introduced into a BalI site (blunt end, lying 564 bp downstream of the start codon of *fscMI*) of pJTU26 to give pJTU38. The 8.0 kb BglII fragment was recovered from pJTU38 and introduced into the BamHI site of pHZ1358 [34] to generate a final construct (pJTU56) used for gene disruption of *fscMI* in the wild-type strain FR-008.

For generating an insertion inside *fscMII*, a 1.4 kb PstI fragment from pHZ1070 (M. Tao, personal communication) carrying the apramycin resistance gene [*acc(3)IV*] was inserted into the corresponding site inside *fscMII* gene (436 bp downstream of its start codon) in pJTU27, a pIJ2925 derivative carrying a 5.5 kb SacI-KpnI fragment carrying *fscMII* to give pJTU31. The 6.9 kb BglII fragment including the insertion was recovered from pJTU31 for ligation into the BamHI site of pHZ1358 to generate pJTU58 for targeted disruption of *fscMII*.

pJTU56 and pJTU58 was transferred by conjugation from *E. coli* ET12567 carrying RP4 derivative pUZ8002 [45, 46] into *Streptomyces* sp. FR-008. Thiostrepton-sensitive and apramycin-resistant ($Thio^S$-Apr^R) colonies (CS101 for *fscMI* mutant and CS102 for *fscMII* mutant, respectively) were counterselected from the initial $Thio^R$ exconjugants after two rounds of nonselective growth.

Five $Thio^S$-Apr^R exconjugants (CS101) were proved to have the

expected gene disruption by PCR amplification using oligonucleotide primers CS3 (5′-GATCCTCTTCGTCAGTCTCC-3′) and CS4 (5′-CATGTAGACCACCGACGACT-3′), whose nucleotide sequences correspond to nt 20–39 and 841–860 downstream of the start codon of *fscMI* (1377 bp), and the amplified PCR product would traverse the BalI restriction site in *fscMI*. Instead of a 840 bp PCR fragment using chromosomal DNA of wild-type strain *Streptomyces* sp. FR-008 as template, a fragment of only 2.2 kb could be seen when chromosomal DNA of CS101 was used as template (data not shown), indicating that the *fscMI* gene had been disrupted by the 1.4 kb apramycin-resistance gene [*acc(3)IV*] in CS101.

Confirmation of the five $Thio^S$-Apr^R exconjugants (CS102) to have the expected *fscMII* gene disruption was performed using oligonucleotide primers CS5 (5′-GACCTGAACATCGACGTCAC-3′) and CS6 (5′-AGGTCGTACATCCACAGGAC-3′), whose nucleotide sequences correspond to nt 315–334 and 804–823 downstream of the start codon of *fscMII* (1059 bp) and amplified PCR product would traverse the PstI restriction site in *fscMII*. Instead of a 508 bp PCR, fragment resulted by using wild-type strain *Streptomyces* sp. FR-008 as template, a fragment of only 1.9 kb could be seen when CS102 was used as template (data not shown), indicating that the *fscMII* gene had been disrupted by the 1.4 kb apramycin-resistance gene [*acc(3)IV*] in CS102.

Deletion of a Contiguous DNA Fragment Carrying Putative Regulatory Genes

A pHZ1358 [34] derivative, pHZ2007, was constructed for gene replacement after a cassette carrying the apramycin [*aac(3)IV*] and erythromycin resistance (*ermE*) genes was sandwiched between a 1518 bp BglII-BamHI DNA fragment (as left arm) and a 1952 bp SacI-BamHI DNA fragment (as right arm), both from pHZ138 [16], to remove a 6414 bp DNA fragment carrying the regulatory genes *fscRII*, *fscRIII*, and a partial *fscRI*. Three $Thio^S$-Apr^R exconjugants (HJ1-1, HJ1-2, and HJ1-3) all had the expected deletion of approximately 6.4 kb, clearly distinguishable by the fusion of the 2.8 kb *aac(3)IV/ermE* gene cassette into the 1.5 kb BglII-BamHI fragment (left arm) of the wild-type FR-008 chromosome. This fusion formed a 4.3 kb BglII-BamHI fragment, as confirmed by Southern hybridization with DIG-dUTP-labeled DNA carrying left (1.5 kb) and right (1.9 kb) arms as well as a *aac(3)IV/ermE* gene cassette (2.8 kb) isolated from pHZ2007 as a probe (data not shown).

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References

1. Gil, J.A., and Martin, J.F. (1995). Polyene antibiotics. In Biotechnology of Antibiotics, W.R. Strohl, ed. (New York: M. Dekker), pp. 551–575.
2. Bolard, J. (1986). How do the polyene macrolide antibiotics affect the cellular membrane properties? Biochim. Biophys. Acta *864*, 257–304.
3. Caffrey, P., Lynch, S., Flood, E., Finnan, S., and Oliynyk, M. (2001). Amphotericin biosynthesis in *Streptomyces nodosus*: deductions from analysis of polyketide synthase and late genes. Chem. Biol. *8*, 713–723.
4. Brautaset, T., Sekurova, O.N., Sletta, H., Ellingsen, T.E., StrLm, A.R., Valla, S., and Zotchev, S.B. (2000). Biosynthesis of the polyene antifungal antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway. Chem. Biol. *7*, 395–403.
5. Aparicio, J.F., Fouces, R., Mendes, M.V., Olivera, N., and Martin, J.F. (2000). A complex multienzyme system encoded by five polyketide synthase genes is involved in the biosynthesis of the 26-membered polyene macrolide pimaricin in *Streptomyces natalensis*. Chem. Biol. *7*, 895–905.
6. Campelo, A.B., and Gil, J.A. (2002). The candicidin gene cluster from *Streptomyces griseus* IMRU 3570. Microbiol. *148*, 51–59.
7. Cortes, J., Haydock, S.F., Roberts, G.A., Bevitt, D.J., and Leadlay, P.F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. Nature *348*, 176–178.
8. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J., and Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. Science *252*, 675–679.
9. MacNeil, T., Gewain, K.M., and MacNeil, D.J. (1993). Deletion analysis of the avermectin biosynthetic genes of *Streptomyces avermitilis* by gene cluster displacement. J. Bacteriol. *175*, 2552–2563.
10. Motamedi, H., and Shafiee, A. (1998). The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK506. Eur. J. Biochem. *256*, 528–534.
11. Schwecke, T., Aparicio, J.F., Molnar, I., Konig, A., Khaw, L.E., Haydock, S.F., Oliynyk, M., Caffrey, P., Cortes, J., Lester, J.B., et al. (1995). The biosynthetic gene cluster for the polyketide immunosuppressant rapamycin. Proc. Natl. Acad. Sci. USA *92*, 7839–7843.
12. August, P.R., Tang, L., Yoon, Y.J., Ning, S., Muller, R., Yu, T.W., Taylor, M., Hoffmann, D., Kim, C.G., Zhang, X., et al. (1998). Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. Chem. Biol. *5*, 69–79.
13. Kuhstoss, S., Huber, M., Turner, J.R., Paschal, J.W., and Rao, R.N. (1996). Production of a novel polyketide through the construction of a hybrid polyketide synthase. Gene *183*, 231–236.
14. Yuan, D., and Zhou, Q. (1991). Studies on breeding of antibiotic-producing strain 5102 by protoplast fusion. IV: Verification of fusant FR-008, and isolation and characterization of the new antimicrobial substance. Chin. J. Biotechnol. *7*, 135–143.
15. Yuan, D., and Zhou, Q. (1990). The killing activity to mosquito larvae of a new antibiotic produced by FR-008, an intra-specific fusant of *S. hygroscopicus var. yingchengensis*. J. Huazhong Agricult. Univ. *9*, 209.
16. Hu, Z., Bao, K., Zhou, X., Zhou, Q., Hopwood, D.A., Kieser, T., and Deng, Z. (1994). Repeated polyketide synthase modules involved in the biosynthesis of a heptaene macrolide by *Streptomyces* sp. FR-008. Mol. Microbiol. *14*, 163–172.
17. Aparicio, J.F., Colina, A.J., Ceballos, E., and Martin, J.F. (1999). The biosynthetic gene cluster for the 26-membered ring polyene macrolide pimaricin. A new polyketide synthase organization encoded by two subclusters separated by functionalization genes. J. Biol. Chem. *274*, 10133–10139.
18. Tang, L., Yoon, Y.J., Choi, C.Y., and Hutchinson, C.R. (1998). Characterization of the enzymatic domains in the modular polyketide synthase involved in rifamycin B biosynthesis by *Amycolatopsis mediterranei*. Gene *216*, 255–265.
19. Haydock, S.F., Aparicio, J.F., Molnar, I., Schwecke, T., Khaw, L.E., Konig, A., Marsden, A.F., Galloway, I.S., Staunton, J., and Leadlay, P.F. (1995). Divergent sequence motifs correlated with the substrate specificity of (methyl)malonyl-CoA:acyl carrier protein transacylase domains in modular polyketide synthases. FEBS Lett. *374*, 246–248.
20. Reid, R., Piagentini, M., Rodriguez, E., Ashley, G., Viswanathan, N., Carney, J., Santi, D.V., Hutchinson, C.R., and McDaniel, R. (2003). A model of structure and catalysis for ketoreductase domains in modular polyketide synthases. Biochemistry *42*, 72–79.
21. Heathcote, M.L., Staunton, J., and Leadlay, P.F. (2001). Role of type II thioesterases: evidence for removal of short acyl chains produced by aberrant decarboxylation of chain extender units. Chem. Biol. *8*, 207–220.
22. Butler, A.R., Bate, N., and Cundliffe, E. (1999). Impact of thioes-

terase activity on tylosin biosynthesis in *Streptomyces fradiae*. Chem. Biol. *6*, 287–292.
23. Tang, L., Fu, H., Betlach, M.C., and McDaniel, R. (1999). Elucidating the mechanism of chain termination switching in the picromycin/methymycin polyketide synthase. Chem. Biol. *6*, 553–558.
24. Kim, B.S., Cropp, T.A., Beck, B.J., Sherman, D.H., and Reynolds, K.A. (2002). Biochemical evidence for an editing role of thioesterase II in the biosynthesis of the polyketide pikromycin. J. Biol. Chem. *277*, 48028–48034.
25. Walsh, C.T., Erion, M.D., Walts, A.E., Delany, J.J., 3rd, and Berchtold, G.A. (1987). Chorismate aminations: partial purification of *Escherichia coli* PABA synthase and mechanistic comparison with anthranilate synthase. Biochemistry *26*, 4734–4745.
26. Ye, Q.Z., Liu, J., and Walsh, C.T. (1990). *p*-Aminobenzoate synthesis in *Escherichia coli*: purification and characterization of PabB as aminodeoxychorismate synthase and enzyme X as aminodeoxychorismate lyase. Proc. Natl. Acad. Sci. USA *87*, 9391–9395.
27. Green, J.M., Merkel, W.K., and Nichols, B.P. (1992). Characterization and sequence of *Escherichia coli pabC*, the gene encoding aminodeoxychorismate lyase, a pyridoxal phosphate-containing enzyme. J. Bacteriol. *174*, 5317–5323.
28. Zielinski, J., Borowy-Borowski, H., Golik, J., Gumieniak, J., Ziminski, T., Kolodzi-Ejczyk, P., Pawlak, J., and Borowski, E. (1979). The structure of levorin A_2 and candicidin D. Tetrahedron Lett. *20*, 1791–1794.
29. Huang, X., Deng, Z., and Liao, R. (1999). Characterization of the heptaene macrolide antibiotic produced by *Streptomyces* sp. FR-008. Chin. J. Antibiot. *24*, 329–333.
30. Omura, S., and Tanaka, H. (1984). Polyene production, structure and activity. In Macrolide Antibiotics, Chemistry, Biology and Practice, S. Omura, ed. (New York: Academic Press), pp. 351–404.
31. Borowski, E., Zielinski, J., Ziminski, T., Falkowski, L., Kolodziejczyk, P., Golik, J., and Jereczek, E. (1970). Chemical studies with amphotericin B. 3. The complete structure of the antibiotic. Tetrahedron Lett. *45*, 3909–3914.
32. Zotchev, S., Haugan, K., Sekurova, O., Sletta, H., Ellingsen, T.E., and Valla, S. (2000). Identification of a gene cluster for antibacterial polyketide-derived antibiotic biosynthesis in the nystatin producer *Streptomyces noursei* ATCC 11455. Microbiol. *146*, 611–619.
33. Bao, K., Hu, Z., Zhou, X., Zhou, Q., and Deng, Z. (1997). A bifunctional cosmid vector for the mobilized conjugal transfer of DNA from *E. coli* to *Streptomyces sp*. Prog. Nat. Sci. *7*, 568–572.
34. Sun, Y., Zhou, X., Liu, J., Bao, K., Zhang, G., Tu, G., Kieser, T., and Deng, Z. (2002). *Streptomyces nanchangensis*, a producer of the insecticidal polyether antibiotic nanchangmycin and the antiparasitic macrolide meilingmycin, contains multiple polyketide gene clusters. Microbiol. *148*, 361–371.
35. Sun, Y., Zhou, X., Dong, H., Tu, G., Wang, M., Wang, B., and Deng, Z. (2003). A complete gene cluster from *Streptomyces nanchangensis* NS3226 encoding biosynthesis of the polyether ionophore nanchangmycin. Chem. Biol. *10*, 431–441.
36. Hanahan, D., and Meselson, M. (1983). Plasmid screening at high colony density. Methods Enzymol. *100*, 333–342.
37. Short, J.M., Fernandez, J.M., Sorge, J.A., and Huse, W.D. (1988). Lambda ZAP: a bacteriophage lambda expression vector with in vivo excision properties. Nucleic Acids Res. *16*, 7583–7600.
38. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
39. Ishikawa, J., and Hotta, K. (1999). FramePlot: a new implementation of the frame analysis for predicting protein-coding regions in bacterial DNA with a high G + C content. FEMS Microbiol. Lett. *174*, 251–253.
40. Schaffer, A.A., Aravind, L., Madden, T.L., Shavirin, S., Spouge, J.L., Wolf, Y.I., Koonin, E.V., and Altschul, S.F. (2001). Improving the accuracy of PSI-BLAST protein database searches with composition-based statistics and other refinements. Nucleic Acids Res. *29*, 2994–3005.
41. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. *25*, 3389–3402.
42. Altschul, S.F., and Koonin, E.V. (1998). Iterated profile searches with PSI-BLAST—a tool for discovery in protein databases. Trends Biochem. Sci. *23*, 444–447.
43. Pearson, W.R. (1990). Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol. *183*, 63–98.
44. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. *22*, 4673–4680.
45. MacNeil, D.J., Occi, J.L., Gewain, K.M., MacNeil, T., Gibbons, P.H., Ruby, C.L., and Danis, S.J. (1992). Complex organization of the *Streptomyces avermitilis* genes encoding the avermectin polyketide synthase. Gene *115*, 119–125.
46. Mazodier, P., Petter, R., and Thompson, C. (1989). Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. *171*, 3583–3585.

Accession Numbers

The complete DNA and deduced protein sequences of the FR-008 biosynthetic gene cluster reported in this paper have been deposited in GenBank under the accession number AY310323.