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Tailoring the P450 Monooxygenase Gene for FR-008/Candicidin Biosynthesis▽†

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Tailoring steps are often important for the activity of mature antibiotics. Here, we report that novel decarboxylated FR-008/candicidin derivatives were obtained from the P450 monooxygenase gene *fscP* mutant of *Streptomyces* sp. strain FR-008. The toxicity of decarboxylated FR-008/candicidin derivatives has been shown to be greatly reduced compared to that of wild-type FR-008/candicidin.

Polyene compounds constitute a large group of effective clinical antifungal antibiotics (3). Polyene antibiotics have severe side effects in patients due to their affinity for sterols in the human body and are especially toxic to the kidney. Therefore, it is important to develop polyene derivatives with inherently reduced toxicity. It is a common phenomenon that the completed polyketide chains are released from the polyketide synthase and undergo further modifications after cyclization, called tailoring steps, including glycosylation, hydroxylation, carboxylation, and epoxylation (2, 4, 6, 13).

FR-008/candicidin (Fig. 1) is useful for the treatment of *Trichomonas vaginalis* infection and has been used for treating moniliform colpitis (8, 10, 12, 15). In addition, FR-008/candicidin can kill mosquito larvae (16), prevent benign prostatic hyperplasia, and has a proven effect on cholesterol and bile acid metabolism (11, 14).

FscP encoded by the FR-008/candicidin gene cluster is highly homologous with its counterparts AmphN, NysN, and PimG of the other polyene pathways (1, 2, 4–8, 13). FscP and FscFE (carrying the electron transfer of ferredoxin in the P450 system) represent a P450 monooxygenase system that is postulated to be responsible for the oxidation of the methyl branch into a carboxyl group, presumably introduced in the 13th elongation step by the methylmalonate-specific AT13 of FscD (Fig. 2). It also remains ambiguous whether an additional oxidoreductase is required to convert a methyl branch into a carboxyl group. Our objective was to inactivate the *fscP* gene as an initial step toward understanding its functional role(s).

Our initial attempt to generate an in-frame deletion of the *fscP* gene resulted in a strain with poor sporulation and low antibiotic yield. We then generated a construct for the targeted replacement of a specific BssHII fragment inside *fscP*, with an apramycin resistance gene on the chromosome of *Streptomyces* sp. strain FR-008 (Fig. 3 and 4). We successfully obtained an *fscP* deletion mutant, CS103, producing novel FR-008/candicidin derivatives.

We next constructed an expression plasmid carrying *fscP-fscFE* and introduced it into CS103. CS103, complemented in this way, restored normal FR-008/candicidin production to a level similar to that of the wild-type *Streptomyces* sp. strain FR-008 (data not shown), which unambiguously confirmed that the deficiency of this specific P450 monooxygenase system accounted for the nonproducing phenotypes in CS103. As an additional control, CS103 was not complemented by the *fscTE* gene lying immediately downstream of *fscP-fscFE*, since FR-008/candicidin production was not recovered and no other phenotypic changes were observed, confirming that the recovered FR-008/candicidin production is *fscP* specific.

Although no FR-008/candicidin was detected by high-performance liquid chromatography analysis, we observed new peaks that possibly represent FR-008/candicidin derivatives in CS103. However, the production levels were only 1% of the level observed with the wild-type producer. By optimizing the production of the novel decarboxylated FR-008/candicidin during submerged fermentation of CS103, the production levels of the same producer improved from 3.33 μg/ml to an optimized level of 60 μg/ml in the 30-liter fermentor (see the supplemental material). This represents a successful combination of the modern genetic breeding of the antibiotic producer with the immediate improvement by traditional fermentation technology.

Analysis of the novel polyene fraction by electrospray ionization mass spectrometry (ESI-MS) revealed two major prod-

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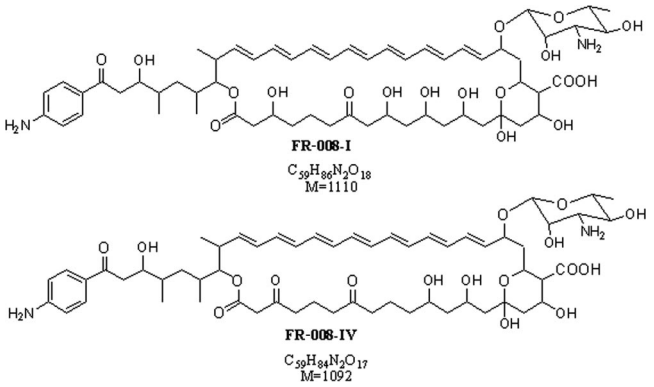

FIG. 1. Chemical structures of FR-008/candicidin. M, molecular weight.

ucts showing absorption spectra characteristic of a heptaene macrolide, with calculated molecular weights of 1,080.5 and 1,062.5, respectively (Fig. 5). The molecular weights of these compounds correspond to the decarboxylated FR-008-I (molecular weight, 1,110.5) and FR-008-IV (molecular weight, 1,092.5), respectively, with a difference in weight of 30 (the difference between the methyl group and the carboxyl group). The two compounds with the same molecular weight, 1,062.5, seem to be isomers (possibly indicating the coexistence of a six-membered cyclic ketalic ring structure with the non-six-membered ketalic ring structure).

We then carried out a mass fragmentation study of the above-mentioned compounds produced by CS103 (Fig. 5). Under the conditions described in our previous studies, the characteristic fragmentation pattern showed the loss of a carboxyl (—COO) moiety (mass, −44). Under the same conditions, we could not detect such a fragmentation pattern (mass, −44) (Fig. 5). Furthermore, the nuclear magnetic resonance spectrum of CS103-I (see Table S4 in the supplemental material) revealed no carboxyl group. This suggests that CS103-I and CS103-II do not harbor a carboxyl group, in agreement with their mass spectra as decarboxylated FR-008/candicidin derivatives.

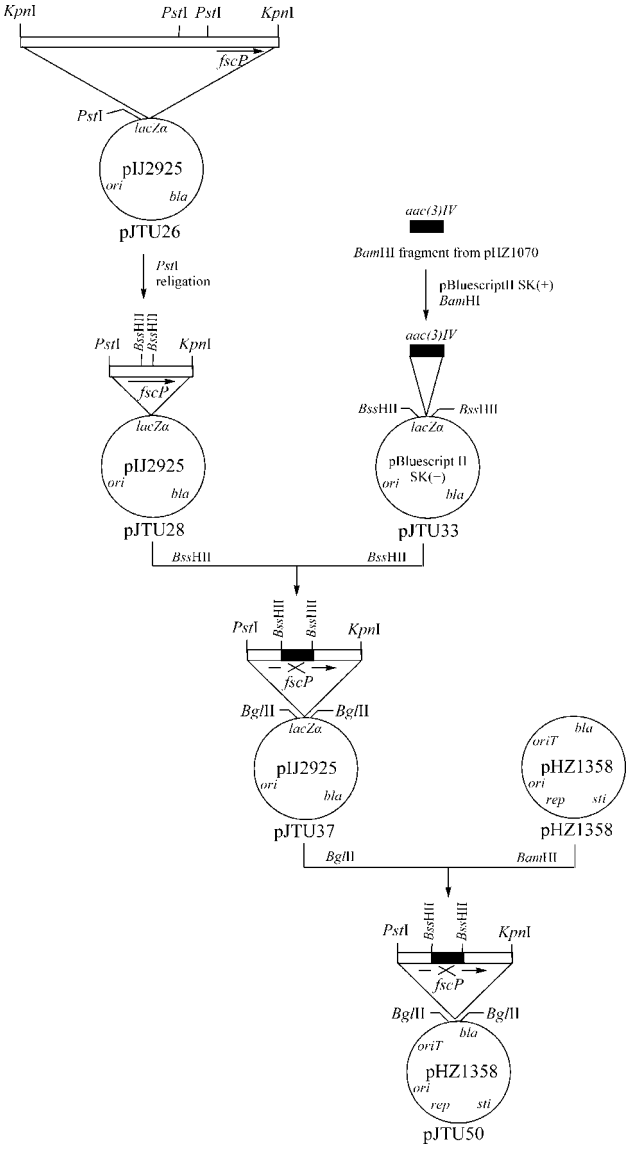

FIG. 3. Schematic of the cloning strategy.

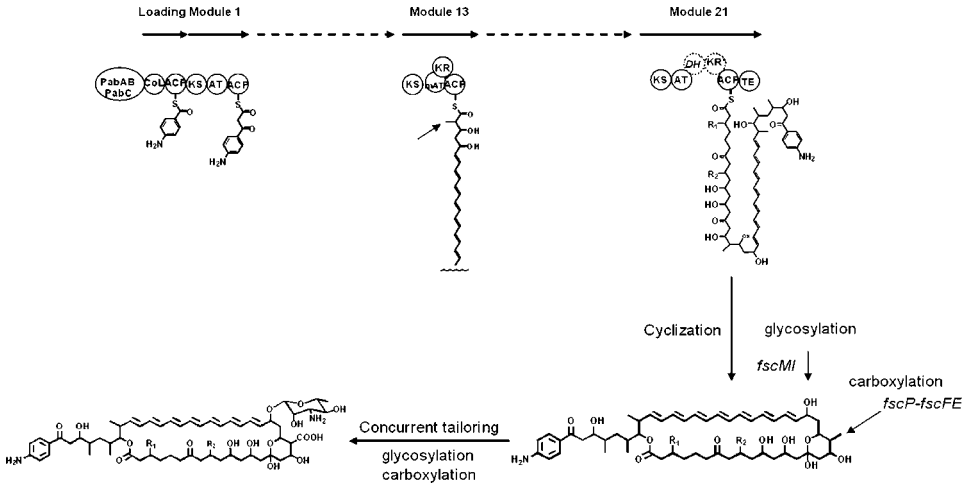

FIG. 2. Proposed metabolic pathway of FR-008/candicidin.

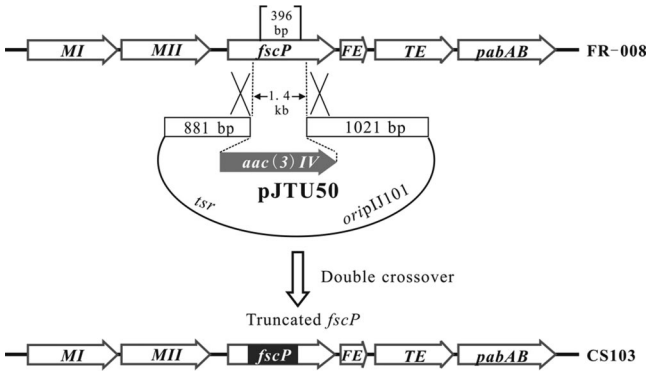

FIG. 4. Deletion of *fscP* by insertion of an apramycin resistance gene [*aac(3)IV*] into the genome of wild-type *Streptomyces* sp. strain FR-008.

The inhibitory activity of the novel decarboxylated FR-008/candicidin to *Saccharomyces cerevisiae* Y029 was compared in parallel with the inhibition zone displayed by the wild-type FR-008/candicidin compound. CS103 showed a MIC of 0.00312 to 0.00625 μg/ml, whereas FR-008/candicidin showed a MIC of 0.00039 to 0.00078 μg/ml. The antifungal activity of decarboxylated FR-008/candicidin is about 5 to 10 times lower than that displayed by the wild-type FR-008/candicidin.

We also measured the toxicity displayed by the decarboxylated candicidin produced by CS103. FR-008/candicidin dispersed in dimethyl sulfoxide caused 50% hemolysis of rabbit erythrocytes at a concentration of ca. 1.5 μg/ml. However, at the highest concentration (74 μg/ml), decarboxylated FR-008/candicidin caused less than 50% hemolysis (Fig. 6), which is approximately 50 times lower than that for FR-008/candicidin.

Modification of the exocyclic carboxyl group of amphotericin B is known to bring about a substantial reduction in its toxicity (9). Interestingly, decarboxylated amphotericin B derivatives lacking exocyclic carboxyl groups also were one to five times less active and 10 to 33 times less hemolytic than amphotericin B (7).

The role of the *fscP* gene in FR-008/candicidin biosynthesis may be as a dioxydase for the introduction of two oxygen atoms into an unactivated methyl group in the macrolide antibiotics modification process, in agreement with the conclusion of Carmody et al. (7). It has been proven that no hydroxylation tailoring exists in FR-008/candicidin biosynthesis (17). When glycosylation with GDP-mycosamine was abolished by targeted disruption of the *fscMI* gene in CS101, the carboxyl group still

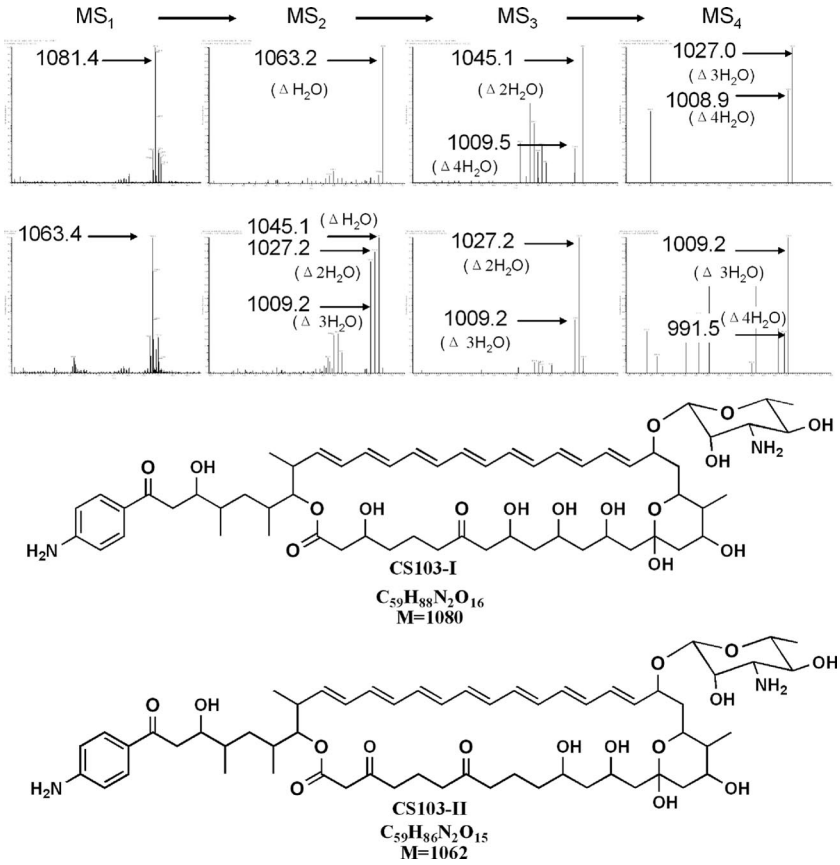

FIG. 5. Analysis of the polyene produced by the *fscP*-interrupted mutant of *Streptomyces* sp. strain FR-008 by EIS-MS and MS_n. CS103-I, 1,080.5 observed molecular weight (M + H = 1,081.5, M + Na = 1,103.5, M + K = 1,119.5); CS103-II, 1,062.5 observed molecular weight (M + H = 1,063.5, M + Na = 1,085.5, M + K = 1,101.5). Characterized loss of a carboxyl (—COO) moiety could not be detected by MS_n analysis. The EIS-MS_1/MS_2/MS_3/MS_4 fragmentation data for each successive step relevant to each of the indicated structures were amplified to show *m*/*z* changes after the loss of specific groups [$(H_2O)_n$]. M, molecular weight.

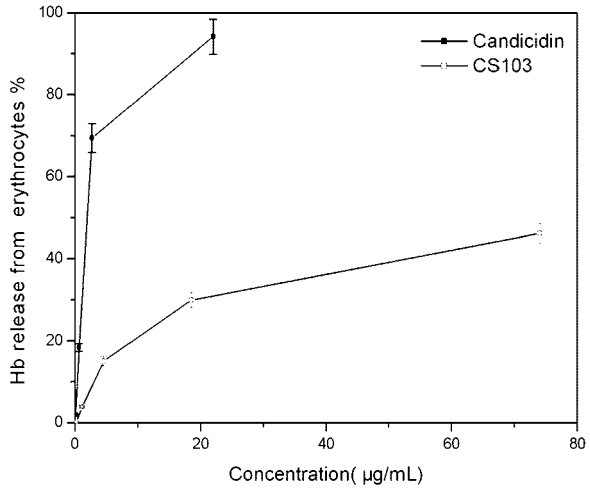

FIG. 6. Dose-response curves obtained for hemolysis of rabbit erythrocytes. Hb, hemoglobin.

formed (8). However, when exocyclic carboxylation was disrupted by targeted inactivation of the *fscP* cytochrome P450 gene, glycosylation still occurred in CS103. We thus conclude that there is no strict order for the tailoring steps in FR-008/candicidin biosynthesis.

Meanwhile, we are attempting to measure the effect of the new derivative compounds on cholesterol and bile acid metabolism (14), in comparison with the effect of wild-type FR-008/candicidin for the further evaluation of its other biological activities and/or potential pharmaceutical applications (Fig. 2).

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