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Function of Cytochrome P450 Enzymes RosC and RosD in the Biosynthesis of Rosamicin Macrolide Antibiotic Produced by *Micromonospora rosaria*

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The cytochrome P450 enzyme-encoding genes *rosC* and *rosD* were cloned from the rosamicin biosynthetic gene cluster of *Micromonospora rosaria* IFO13697. The functions of RosC and RosD were demonstrated by gene disruption and complementation with *M. rosaria* and bioconversion of rosamicin biosynthetic intermediates with *Escherichia coli* expressing RosC and RosD. It is proposed that *M. rosaria* IFO13697 has two pathway branches that lead from the first desosaminyl rosamicin intermediate, 20-deoxo-20-dihydro-12,13-deepoxyrosamicin, to rosamicin.

Oxidation catalyzed by cytochrome P450 enzymes in post-polyketide synthase (post-PKS) modification of macrolide antibiotics contributes to structural diversification and modulates bioactivity. Rosamicin, which is a 16-member macrolide antibiotic produced by *Micromonospora rosaria* IFO13697 (1), contains an epoxide and a formyl group at the C-12/13 and C-20 positions, respectively, and it is expected that two different types of P450s generate these functional groups (Fig. 1). Recently, we reported that the mycinosyl rosamicin derivatives were produced by genetic engineering of *M. rosaria* TPMA0001 (2, 3). Here, we cloned the cytochrome P450 enzyme-encoding genes *rosC* and *rosD* from *M. rosaria* IFO13697 and demonstrated the functions of RosC and RosD in the rosamicin biosynthetic pathway.

Six complete protein-coding regions (*orf1* to *orf4*, *rosC*, and *rosD*) and a partial protein-coding region (*rosAI*) were contained in the 9,036-bp DNA fragment in the cosmid pRS85, which was isolated using a PCR product amplified with degenerate primers as a DNA probe for colony hybridization. The primers were designed from two conserved regions of deduced amino acid sequences of P450s implicated in formylation of 16-member macrolides (4–8). The complete nucleotide sequence of the rosamicin biosynthetic gene cluster in *Micromonospora carbonacea* subsp. *aurantiaca* NRRL2997 was determined by Farnet et al. (8). The deduced amino acid sequences of RosC and RosD were most similar to P450s encoded in ORF3 and ORF4 of *M. carbonacea* subsp. *aurantiaca* NRRL 2997 (87% and 83% identity, respectively) (see Fig. S1 in the supplemental material). In BLAST searches, RosC and RosD were similar to TylI (71% identity) in tylosin biosynthesis and OleP (48% identity) in oleandomycin biosynthesis, respectively (4, 9).

To obtain the *rosC* and *rosD* disruption mutants of *M. rosaria* IFO13697, disruption plasmids pRS511 and pRS514 were constructed using a PCR-targeting method (10). These disruption plasmids were introduced into *M. rosaria* IFO13697 by conjugation using our previous procedure (2). The resulting disruption mutants, TPMA0050 and TPMA0055, did not produce rosamicin when the strains were cultured in 172F medium. However, an unknown compound, RS-B, accumulated in the TPMA0050 culture broth, and unknown peaks RS-C, RS-D, and RS-E were detected in ethyl acetate extract of the TPMA0055 culture broth by high-performance liquid chromatography (HPLC) analysis (Fig. 2; see Fig. S2 in the supplemental material). Furthermore, when *rosC* disruption plasmid pRS516 was introduced into TPMA0055 (Δ*rosD*), the resulting *rosC rosD* double-disruption mutant TPMA0063 (Apr^r) accumulated RS-E in the culture broth (Fig. 2; see Fig. S2). RS-B (12.8 mg) and RS-E (5.1 mg) were isolated and purified from 1.8 liters of culture broth of TPMA0050 and TPMA0063, respectively. RS-C (8.9 mg) and RS-D (5.8 mg) were isolated and purified from 112 × 15 ml MR0.1S culture plates of TPMA0055. Based on nuclear magnetic resonance (NMR) chemical shifts (see Tables S3, S4, and S5 in the supplemental material), mass spectrometry (MS) data (RS-B, *m/z* 567; RS-C, *m/z* 567; RS-D, *m/z* 565; RS-E, *m/z* 551), and the UV absorption spectrum, the structures of RS-B, RS-C, RS-D, and RS-E were determined to be 20-deoxo-20-dihydrorosamicin, 20-dihydro-12,13-deepoxyrosamicin, 12,13-deepoxyrosamicin, and 20-deoxo-20-dihydro-12,13-deepoxyrosamicin, respectively (Fig. 1) (1, 11). The antibacterial activities of RS-D and rosamicin (with a formyl group at C-20) were higher than those of the other rosamicin biosynthetic intermediates (see Table S6 in the supplemental material). To construct pRS518 and pRS519 for genetic complementation of the *rosC* and *rosD* disruption mutants, a 2.0-kb NruI fragment, including *rosC*, and a 3.0-kb EcoRV-BglII fragment, including *rosD*, were inserted into the site-specific integration vector pSET152 (12), which could be integrated into the ϕC31 *attB* site on the chromosome of *M. rosaria* IFO13697 by the ϕC31 *att*/*int* system (2). These plasmids were introduced into TPMA0050 and TPMA0055. The resulting transconjugants, TPMA0053 and TPMA0066, restored the productivity of rosamicin; however, the amount of rosamicin produced by TPMA0053 and TPMA0066 was smaller than that produced by the wild strain IFO13697. RS-A, which was not detected in the culture broth of

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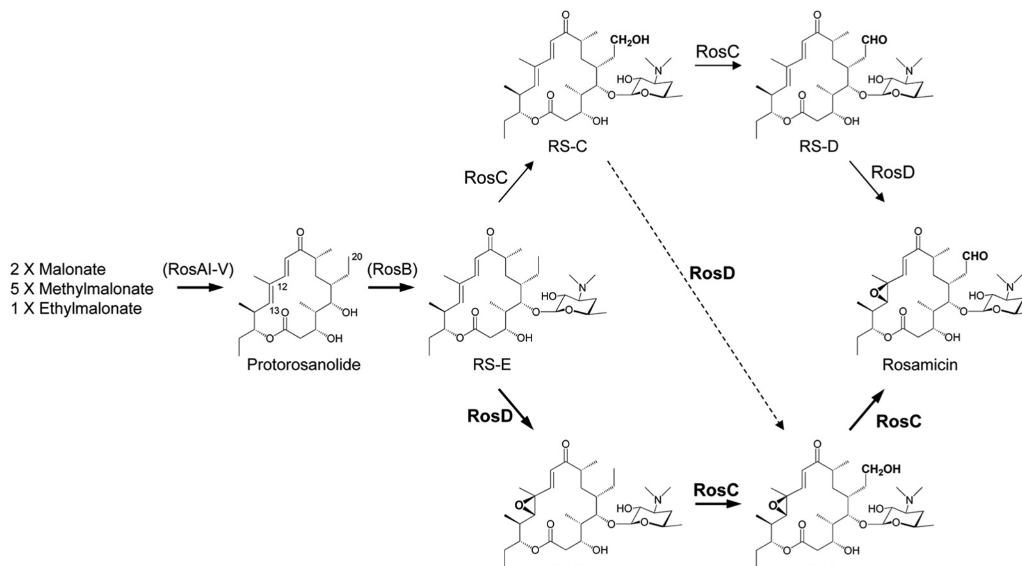

FIG 1 The proposed biosynthetic pathway of rosamicin in *M. rosaria* IFO13697. The bold arrows represent the main pathway investigated in this study. The dashed arrow indicates the reaction with *E. coli* TPMB0003 cells expressing RosD. RosAI-V, putative polyketide synthase; RosB, putative glycosyltransferase; protorosanolide, putative macrolactone of rosamicin (tylactone) (15).

TPMA0050, accumulated in the TPMA0053 culture broth. RS-A (8.4 mg) was isolated and purified from 1.8 liters of TPMA0053 culture broth, and the structure of RS-A was determined from the NMR shifts (see Table S3 in the supplemental material), MS data (*m/z* 583), and UV absorption spectrum to be a rosamicin intermediate, 20-dihydrorosamicin (1, 11).

To elucidate the biosynthetic pathway from RS-E to rosamicin, bioconversions of rosamicin biosynthetic intermediates were performed using a bacterial P450 expression system (13) with *E. coli* TPMB0002 and TPMB0003, which were expressing RosC and RosD, respectively. The first desosaminyl rosamicin intermediate, RS-E, was recognized as a substrate of RosC and RosD. RS-C and RS-D were detected in the reaction mixture of RS-E and TPMB0002, and RS-E was converted into RS-B by TPMB0003 (Table 1). Moreover, RS-C and RS-D, with a double bond at C-12/13, were converted into RS-A and rosamicin, respectively, by TPMB0003. Thus, it was confirmed that RosD catalyzes the epoxidation of the C-12/13 double bond of the macrolactone. On the

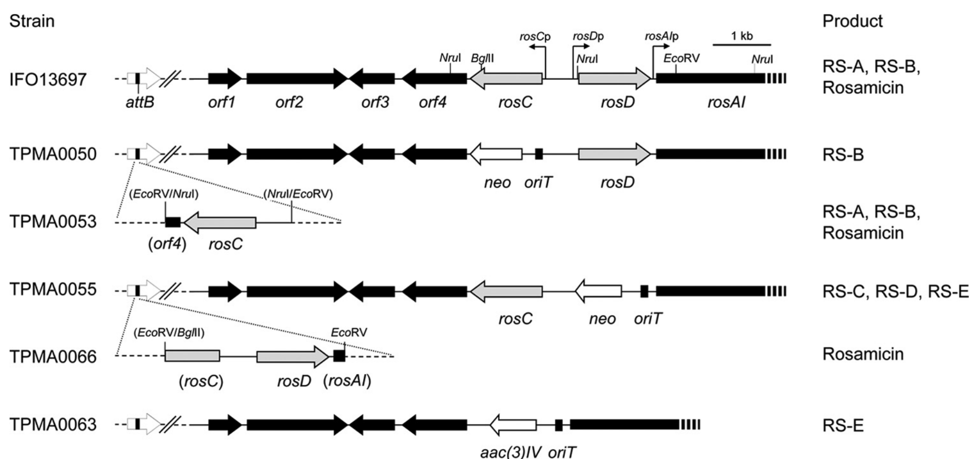

FIG 2 Physical maps of the region that includes *rosC*, *rosD*, and the flanking genes of the wild strain *Micromonospora rosaria* IFO13697, *rosC* disruption mutant TPMA0050, *rosC* complementation strain TPMA0053, *rosD* disruption mutant TPMA0055, *rosD* complementation strain TPMA0066, and *rosC rosD* double-disruption mutant TPMA0063. The strains, plasmids, and PCR primers used in this study are shown in Tables S1 and S2 in the supplemental material. The major products in the culture broth of the wild strain, disruption mutants, and complementation were detected by HPLC (see Fig. S2 in the supplemental material). Rosamicin and its biosynthetic intermediates in the ethyl acetate extracts from the culture broth were analyzed by HPLC. Disruption of the target gene and introduction of the complementation gene were confirmed by Southern blotting and PCR, as described in our previous report (16; data not shown). The *attB* site lies within the ORF of the pirin homolog on the chromosome of *M. rosaria* IFO13697 (2). The gene names and their products or functions and promoter abbreviations are as follows: *orf1*, nucleosidase; *orf2*, ABC transporter; *orf3*, type II thioesterase; *orf4*, aminotransferase; *rosC*, cytochrome P450; *rosD*, cytochrome P450; *rosAI*, type I polyketide synthase; *neo*, neomycin resistance gene; *aac(3)IV*, apramycin resistance gene; *oriT*, origin of transfer from plasmid RP4 (12); *rosC*p, promoter of *rosC*; *rosD*p, promoter of *rosD*; *rosAI*p, promoter of *rosAI*.

TABLE 1 Bioconversion products from rosamicin biosynthetic intermediates with *E. coli* cells expressing RosC and RosD[a]

Intermediate[b]	Bioconversion product(s) of *E. coli* strain[c]:		
	TPMB0002 (RosC)	TPMB0003 (RosD)	TPMB0001 (negative control)
RS-A	RS-A	RS-A	RS-A
RS-B	RS-A, RS-B, rosamicin, unknown compound 1	RS-B	RS-B
RS-C	RS-C	RS-A	RS-C
RS-D	Unknown compound 2	Rosamicin	RS-D
RS-E	RS-C, RS-D, RS-E	RS-B, RS-E	RS-E

[a] The products were detected by HPLC analysis (see Fig. S3 in the supplemental material).
[b] Rosamicin intermediates (40 μg/ml) were added to 1 ml of the cell suspension containing *E. coli* TPMB0001 (negative control), TPMB0002 (RosC), and TPMB0003 (RosD).
[c] Plasmids pCYP-camAB (P450 protein expression vector), pRSC-camAB (pCYP-camAB plus *rosC*), and pRSD-camAB (pCYP-camAB plus *rosD*) were introduced into *E. coli* BL21(DE3). The strains, plasmids, and PCR primers used in this study are shown in Tables S1 and S2 in the supplemental material.

other hand, RosC may catalyze a 3-step hydroxylation-formylation-carboxylation reaction at C-20 on the macrolactone. RS-A and rosamicin were detected in the reaction mixture of RS-B and TPMB0002, and unknown compound 1 was detected in the mixture. When RS-D was incubated with TPMB0002, unknown compound 2 was detected. Mass peaks of these unknown compounds were shown at *m/z* 598 ($M+H^+$) and *m/z* 582 ($M+H^+$) by liquid chromatography (LC)-MS analysis. It was thus predicted that the unknown compounds 1 and 2 were oxidation products of rosamicin and RS-D, respectively, and these compounds have a carboxyl group at C-20 of the macrolactone because 20-carboxyrosamicin was isolated from the fermentation broth of *M. rosaria* (1). TylI likely catalyzes the oxidation of an aldehyde to a carboxylic acid in hybrid 16-member macrolide antibiotic biosynthesis in an engineered strain of *Streptomyces fradiae* (14). In contrast, RS-A and RS-C were not converted into any other derivative by TPMB0002, and it was thus predicted that hydroxylation and dehydrogenation were performed sequentially by RosC to form the formyl group at C-20. This prediction would be confirmed with an *in vitro* binding assay between purified RosC protein and rosamicin intermediates.

The first desosaminyl rosamicin intermediate, RS-E, accumulated in the culture broth of TPMA0063, and both RosC and RosD could recognize RS-E as a substrate in bioconversion studies. Four intermediates—RS-A, RS-B, RS-C, and RS-D—were identified in *rosC* and *rosD* disruption and complementation studies. However, RS-C and RS-D were not detected in the culture broth of the wild strain IFO13697, and RS-A and RS-B accumulated with rosamicin in the broth. Moreover, RS-E was detected with RS-C and RS-D in the culture broth of TPMA0055; however, only RS-B accumulated in the culture broth of TPMA0050. From these results, we propose that *M. rosaria* IFO13697 has two pathways from RS-E to rosamicin in the rosamicin post-PKS biosynthetic pathway, and the main pathway is the conversion of RS-E to RS-B by epoxidase RosD, followed by two-step oxidation (hydroxylation and dehydrogenation) by RosC (Fig. 1).

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REFERENCES

1. **Puar MS, Schumacher D.** 1990. Novel macrolides from *Micromonospora rosaria*. J. Antibiot. **43**:1497–1501.
2. **Anzai Y, Iizaka Y, Li W, Idemoto N, Tsukada S, Koike K, Kinoshita K, Kato F.** 2009. Production of rosamicin derivatives in *Micromonospora rosaria* by introduction of D-mycinose biosynthetic gene with ϕC31-derived integration vector pSET152. J. Ind. Microbiol. Biotechnol. **36**: 1013–1021.
3. **Anzai Y, Sakai A, Li W, Iizaka Y, Koike K, Kinoshita K, Kato F.** 2010. Isolation and characterization of 23-*O*-mycinosyl-20-dihydro-rosamicin: a new rosamicin analogue derived from engineered *Micromonospora rosaria*. J. Antibiot. **63**:325–328.
4. **Merson-Davies LA, Cundliffe E.** 1994. Analysis of five tylosin biosynthetic genes from the *tylIBA* region of the *Streptomyces fradiae* genome. Mol. Microbiol. **13**:349–355.
5. **Schell U, Haydock SF, Kaja AL, Carletti I, Lill RE, Read E, Sheehan LS, Low L, Fernandez MJ, Grolle F, McArthur HA, Sheridan RM, Leadlay PF, Wilkinson B, Gaisser S.** 2008. Engineered biosynthesis of hybrid macrolide polyketides containing D-angolosamine and D-mycaminose moieties. Org. Biomol. Chem. **6**:3315–3327.
6. **Karray F, Darbon E, Oestreicher N, Dominguez H, Tuphile K, Gagnat J, Blondelet-Rouault MH, Gerbaud C, Pernodet JL.** 2007. Organization of the biosynthetic gene cluster for the macrolide antibiotic spiramycin in *Streptomyces ambofaciens*. Microbiology **153**:4111–4122.
7. **Cong L, Piepersberg W.** 2007. Cloning and characterization of genes encoded in the dTDP-D-mycaminose biosynthetic pathway from a midecamycin-producing strain, *Streptomyces mycarofaciens*. Acta Biochim. Biophys. Sin. **39**:187–193.
8. **Farnet CM, Staffa A, Yang X.** June 2003. Genes and proteins for the biosynthesis of rosaramicin. Patent WO **03010193-A**:39.
9. **Rodriguez AM, Olano C, Mendez C, Hutchinson CR, Salas JA.** 1995. A cytochrome P450-like gene possibly involved in oleandomycin biosynthesis by *Streptomyces antibioticus*. FEMS Microbiol. Lett. **127**:117–120.
10. **Gust B, Challis GL, Fowler K, Kieser T, Chater KF.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. U. S. A. **100**:1541–1546.
11. **Kinumaki A, Harada KI, Suzuki T, Suzuki M, Okuda T.** 1977. Macrolide antibiotics M-4365 produced by *Micromonospora*. II. Chemical structures. J. Antibiot. **30**:450–454.
12. **Bierman M, Logan R, O'Brien K, Seno ET, Rao RN, Schoner BE.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces spp*. Gene **116**:43–49.
13. **Agematu H, Matsumoto N, Fujii Y, Kabumoto H, Doi S, Machida K, Ishikawa J, Arisawa A.** 2006. Hydroxylation of testosterone by bacterial cytochromes P450 using the *Escherichia coli* expression system. Biosci. Biotechnol. Biochem. **70**:307–311.
14. **Reeves CD, Ward SL, Revill WP, Suzuki H, Marcus M, Petrakovsky OV, Marquez S, Fu H, Dong SD, Katz L.** 2004. Production of hybrid 16-membered macrolides by expressing combinations of polyketide synthase genes in engineered *Streptomyces fradiae* hosts. Chem. Biol. **11**:1465–1472.
15. **Baltz RH, Seno ET, Stonesifer J, Wild GM.** 1983. Biosynthesis of the macrolide antibiotic tylosin. A preferred pathway from tylactone to tylosin. J. Antibiot. **36**:131–141.
16. **Tsukada S, Anzai Y, Li S, Kinoshita K, Sherman DH, Kato F.** 2010. Gene targeting for *O*-methyltransferase genes, *mycE* and *mycF*, on the chromosome of *Micromonospora griseorubida* producing mycinamicin with a disruption cassette containing the bacteriophage ϕC31 *attB* attachment site. FEMS Microbiol. Lett. **304**:148–156.