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 desosamyl rosamicin intermediate, 20-deoxy-20-dihydro-12,13-deepoxyrosamycin, 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 NRR2997 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 NRR2 997 (87% and 83% identity, respectively) (see Fig. S1 in the supplemental material). In BLAST searches, RosC and RosD were similar to TyII (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 (ΔrosD), the resulting rosC rosD double-disruption mutant TPMA0063 (Apr+) 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-deoxy-20-dihydrorosamicin, 20-dihydro-12,13-deepoxyrosamicin, 12,13-deepoxyrosamicin, and 20-deoxy-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 pRSS518 and pRSS519 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 ΔC31 attB site on the chromosome of M. rosaria IFO13697 by the ΔC31 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...
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 1](https://example.com/fig1.png) 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. RosAl-V, putative polyketide synthase; RosB, putative glycosyltransferase; protorosanolide, putative macrolactone of rosamicin (tylactone) (15).

![FIG 2](https://example.com/fig2.png) 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); rosCp, promoter of rosC; rosDp, promoter of rosD; rosAlp, promoter of rosAl.
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). TyII 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 rosamin post-PKS biosynthetic pathway, and the main pathway is the conversion of RS-E to RS-B by epoxide RosD, followed by two-step oxidation (hydroxylation and dehydrogenation) by RosC (Fig. 1).

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**REFERENCES**


