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-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, 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 (rosA) 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 NRRRL2997 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 NRRRL 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 (ΔrosD), the resulting rosC rosD double-disruption mutant TPMA0063 (Apr⁰) 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 x 15 ml MR0.15 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-dihydrososamycin, 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 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
TABLE 1 Bioconversion products from rosamicin biosynthetic intermediates with E. coli cells expressing RosC and RosDa

<table>
<thead>
<tr>
<th>Intermediate</th>
<th>Bioconversion product(s) of E. coli strainb</th>
<th>TPMB0002 (RosC)</th>
<th>TPMB0003 (RosD)</th>
<th>TPMB0001 (negative control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RS-A</td>
<td>RS-A</td>
<td>RS-A</td>
<td>RS-A</td>
<td></td>
</tr>
<tr>
<td>RS-B</td>
<td>RS-A, RS-B, rosamicin, unknown compound 1</td>
<td>RS-B</td>
<td>RS-B</td>
<td></td>
</tr>
<tr>
<td>RS-C</td>
<td>RS-C</td>
<td>RS-A</td>
<td>RS-C</td>
<td></td>
</tr>
<tr>
<td>RS-D</td>
<td>Unknown compound 2</td>
<td>Rosamicin</td>
<td>RS-D</td>
<td></td>
</tr>
</tbody>
</table>

a The products were detected by HPLC analysis (see Fig. 53 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 pRS-D-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.

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REFERENCES