Function of cytochrome P450 enzymes RosC and RosD in the biosynthesis of rosamicin macrolide antibiotic produced by *Micromonospora rosaria*

Yohei Iizaka, Noriko Higashi, Masanari Ishida, Reina Oiwa, Yumi Ichikawa, Moeka Takeda, Yojiro Anzai*, and Fumio Kato

Faculty of Pharmaceutical Sciences, Toho University, Funabashi, Chiba, Japan

* Corresponding author.

Mailing address: Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan.

Phone: 81 47 472 2074.

Fax: 81 47 472 2086.

E-mail: yanzai@phar.toho-u.ac.jp.
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 was 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 modification of macrolide antibiotics contributes to structural diversification and modulates bioactivity. Rosamicin, which is a 16-membered macrolide antibiotic produced by *M. 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 2 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*-*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 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-membered macrolides (4, 5, 6, 7, 8). The complete nucleotide sequence of the rosamicin biosynthetic gene cluster in *Micromonospora carbonacea* var. *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* var. *aurantiaca* NRRL 2997 (87% and 83% identity, respectively) (see Fig. S1). 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). Furthermore, when *rosC* disruption plasmid pRS516 was introduced into TPMA0055 (Δ*rosD*), the resulting *rosC* *rosD* double disruption mutant TPMA0063 (Aprr) 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 L 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 NMR chemical shifts (see Table S3, S4, S5), MS data (RS-B: *m/z* 567, RS-C: *m/z* 567, RS-D: *m/z* 565, RS-E: *m/z* 551), and 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). To construct pRS518 and pRS519 for genetic complementation of the *rosC* and *rosD* disruption mutants, 2.0-kb NruI fragment including *rosC* and 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 lower than that produced by 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 L TPMA0053 culture broth and the structure of RS-A was determined from the NMR shifts (see Table S3), 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 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 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-membered macrolide antibiotic biosynthesis in an engineered strain of *S. 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 *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 biocconversion 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 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 2 pathways from RS-E to rosamicin in the rosamicin post-PKS biosynthetic pathway, and the main pathway is that RS-E is converted to RS-B by epoxidase RosD and followed by two step oxidation (hydroxylation and dehydrogenation) by RosC (Fig. 1).
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REFERENCES


A. 100:1541-1546.


FIGURE LEGENDS

**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).

**FIG 2** Physical maps of the region, including *rosC*, *rosD* and the flanking genes of the wild type strain *Micromonospora rosaria* IFO13697, *rosC* disruption mutant TPMA0050, *rosC* complementation strain TPMA0053, *rosD* disruption mutant TPMA0055, *rosD* complementation strain TPMA0066, and *rosC* and *rosD* double disruption mutant TPMA0063. The strains, plasmids, and PCR primers used in this study were shown in Table S1 and S2. The major products in the culture broth of the wild strain, disruption mutants, and complementation were detected HPLC (see Fig. S2). Rosamicin and its biosynthetic intermediates in the ethyl acetate extracts from the culture broth were analyzed with HPLC. Disruption of target gene and introduction of complementation gene were confirmed by Southern blot analysis and PCR as described in our previous report (16) (data not shown). The *attB* site lies within the ORF of pirin homolog on the chromosome of *M. rosaria* IFO13697 (2). *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*; *rosAIp*, promoter
of *rosA*.
FIG 1

2 X Malonate
5 X Methylmalonate
1 X Ethylmalonate

Protorosanolide

RosC

RS-C

RosC

RS-D

RosD

RS-E

RosC

RS-B

RosD

RS-A

Rosamicin

RosB

(RosAI-V)

RosD
TABLE 1 Bioconversion products\textsuperscript{a} from rosamicin biosynthetic intermediates with \textit{E. coli} cells expressing RosC and RosD.

<table>
<thead>
<tr>
<th>Intermediate\textsuperscript{c}</th>
<th>Strain\textsuperscript{b}</th>
<th>Strain\textsuperscript{b}</th>
<th>Strain\textsuperscript{b}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>TPMB0002 (RosC)</td>
<td>TPMB0003 (RosD)</td>
<td>TPMB0001 (negative control)</td>
</tr>
<tr>
<td>RS-A</td>
<td>RS-A</td>
<td>RS-A</td>
<td>RS-A</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>
</tr>
<tr>
<td>RS-C</td>
<td>RS-C</td>
<td>RS-A</td>
<td>RS-C</td>
</tr>
<tr>
<td>RS-D</td>
<td>Unknown compound 2</td>
<td>Rosamicin</td>
<td>RS-D</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The products were detected by HPLC analysis (see Fig. S3).

\textsuperscript{b} Plasmids pCYP-camAB (P450 protein expression vector), pRSC-camAB (pCYP-camAB + \textit{ros}C), and pRSD-camAB (pCYP-camAB + \textit{ros}D) were introduced into \textit{E. coli} BL21 (DE3). The strains, plasmids, and PCR primers used in this study were shown in Table S1 and S2.

\textsuperscript{c} Rosamicin intermediates (40 \textmu g/mL) were added to 1 mL of the cell suspension containing \textit{E. coli} TPMB0001 (negative control), TPMB0002 (RosC), and TPMB0003 (RosD).