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ORIGINAL ARTICLE

Monooxygenation of rifampicin catalyzed by the *rox* gene product of *Nocardia farcinica*: structure elucidation, gene identification and role in drug resistance

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We demonstrated that the *rox* gene of *Nocardia farcinica* encodes a rifampicin monooxygenase capable of converting rifampicin to a new compound 2′-*N*-hydroxy-4-oxo-rifampicin with a markedly lowered antibiotic activity. The deletion mutation (Δ*rox*) of the *rox* gene gave no significant influence to the rifampicin resistance of *N. farcinica*. However, transformation with a plasmid containing an overexpressing the *rox* gene markedly raised the rifampicin resistance in the strain with the deletion mutation of the *rpoB2* gene as the principal rifampicin resistance determinant. On the other hand, rifampicin was decolorized by the wild-type strain, whereas it remained intact when incubated with the Δ*rox* strain. Based on these results, it will be conclusive that the *rox* gene is capable of initiating rifampicin degradation with a new metabolite formation at the first step and having a role as the secondary rifampicin resistance factor in *N. farcinica*.
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Keywords: drug resistance; monooxygenase; *Nocardia farcinica*; rifampicin; *rox*

INTRODUCTION

Nocardia species are naturally resistant to wide varieties of antibiotics, for example β-lactams, aminoglycosides, macrolides and rifamycins. Similar to mycobacteria, the hydrophobic nature of the nocardial cell-wall was once believed to act as a permeability barrier for the multidrug resistance of this organism. However, genome sequence analysis of *Nocardia farcinica* IFM 10152 has since revealed the existence of specific genes which explain resistance to each drug.[1] As to rifampicin (RIF), the *rpoB2* gene, a duplicated copy of the RNA polymerase β-subunit gene containing RIF-resistant amino acid substitutions, has been predicted to be a resistance determinant and its contribution to the RIF resistance of *N. farcinica* has been demonstrated experimentally.[2] On the other hand, decolorization of RIF by *N. farcinica* has been reported[3,4] and the sequenced strain IFM 10152 decolorizes RIF in prolonged culture (unpublished observation). This observation implies the participation of decolorization in RIF resistance.

A variety of RIF-modifying enzymes has been identified in *Nocardia* and related genera, such as enzymes involved in phosphorylation,[5] glycosylation[4] and ribosylation.[6] Therefore, in addition to the *rpoB2* gene, we searched the *N. farcinica* genome for genes which might participate in RIF resistance and *rox* (formerly denoted as *nfa35380*) turned out to be a homologous gene to the *iri* gene that confers a low-level resistance to RIF in *Rhodococcus equi*.[7] The deduced amino acid sequence of *rox* (473 aa) shows 64% identity and 77% similarity to that of *iri* (479 aa), thus suggesting that they share similar enzymatic activities. The Iri protein has been believed to be a monooxygenase, attacking the naphthalenyl moiety of RIF, based on its sequence similarity to many monooxygenases acting on phenolic compounds. However, the reaction product of the Iri protein has never been identified so there is no evidence for its proposed action as a monooxygenase. In this study, we determined the chemical structure of the reaction product of Rox protein to discuss plausible functions of *rox* in resistance to and decolorization of RIF.

MATERIALS AND METHODS

Bacterial strains and plasmids

N. farcinica IFM 10152 was obtained from the Medical Mycology Research Center, Chiba University, Japan, and maintained in our laboratory. *Escherichia coli* JM109 or BL21(DE3) was used as a host strain for gene cloning and expression, respectively. pKNL027_N05, a plasmid from the *N. farcinica* IFM 10152 ordered plasmid library (http://nocardia.nih.go.jp/), was used as the source of the *rox* gene fragment. pNV118 (unpublished), a high copy-number

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version of pNV18,[8] was used for the expression of *rox* in *Nocardia* strains. pK18*mobsacB*[9] was obtained from the National Institute of Genetics, Shizuoka, Japan and used for making deletion mutants.

Culture conditions

All strains were incubated at 37 °C unless otherwise indicated. *Nocardia* strains were grown in the brain–heart infusion (BHI) broth (Becton, Dickinson, Franklin Lakes, NJ, USA) or in a minimal medium (MM) consisting of $Na_2HPO_4{\cdot}12H_2O$ 1.5%, KH_2PO_4 0.3%, sodium citrate 0.05%, NH_4Cl 0.02%, $MgSO_4{\cdot}7H_2O$ 0.1%, NaCl 0.05% and glucose 1.5%. *E. coli* strains were grown in the Luria–Bertani (LB) broth. Minimum inhibitory concentrations (MICs) were determined by a standard microdilution method. For *Nocardia* strains, the MICs were scored after 48 h of incubation in BHI broth. For other bacterial strains, MICs were scored after 24 h of incubation in Mueller–Hinton II broth (Becton, Dickinson).

Recombinant DNA techniques

A BigDye Terminator v3.1 cycle sequencing kit and a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) were used for nucleotide sequencing, in accordance with the manufacturer's instructions. PCR was carried out in 10 µl reaction mixtures with a KOD-Plus-kit (TOYOBO, Osaka, Japan). Bacterial cells were used as templates for the screening of transformants by PCR.[10] The PCR amplification program consisted of one cycle of 3 min at 98 °C, followed by 30 cycles of 20 s at 98 °C, 20 s at 55–60 °C and 20 s at 68 °C, with a final extension step at 68 °C for 5 min.

Construction of *rox* expression plasmid

A 1.6-kb DNA fragment containing *rox* (Figure 1) was amplified from pKNL027_N05 by PCR with the use of the primers 35380F (CGCTCTAGAGGT GATCGATGTGATCATCG) and 35380R (ATATCTAGATCAGGTGGCGGCGCC GAAC). The amplified fragment was cloned in-frame at the *Xba*I site of pUC18 and its nucleotide sequence checked, obtaining pUC18*rox*.

Conversion of RIF using *E. coli* resting cells

E. coli BL21(DE3)/pUC18*rox* was grown in 1 l of LB broth containing 50 µg ml^{-1} of ampicillin for 24 h at 27 °C. Cells were washed with sterile water and suspended in 500 ml of 100 mM potassium phosphate buffer (pH 7.0), supplemented with 1% glucose. RIF was added to the cell suspension at a final concentration of 100 µg ml^{-1} and incubated for 24 h at 27 °C with vigorous shaking. Cells were removed by centrifugation and the reaction products were recovered from the buffer by adsorption on DIAION HP20 resin (Mitsubishi Chemical, Tokyo, Japan). The resin was washed with water and the reaction products were eluted with methanol. These procedures were repeated and the reaction products were pooled. To monitor the reaction, thin-layer chromatography (TLC) was performed on a silica gel plate (silica gel $60F_{254}$, Merck & Co., Whitehouse Station, NJ, USA) using a chloroform:methanol:water (65:15:5, lower phase) solvent system.

Purification of reaction products

The pooled reaction products were purified by silica gel-column chromatography (silica gel 60; Nacalai Tesque, Kyoto, Japan) using stepwise elution with chloroform containing increasing amounts of methanol (1:0, 30:1, 20:1, 15:1, 10:1, 5:1 and 1:1). The fractions (10:1 and 5:1) containing the main compound were pooled and applied to a preparative TLC plate (silica gel $60F_{254}$, 0.5 mm thick, Merck & Co.) and developed using a chloroform:methanol:water (65:15:5, lower phase) solvent system. The isolated fraction was further purified by Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) column chromatography using methanol as an elution solvent. The final yield was 50 mg of purified product.

Physicochemical analyses

The ^{1}H-, ^{13}C- and 2D-NMR (^{1}H–^{1}H COSY, heteronuclear multiple quantum coherence (HMQC), ^{1}H–^{13}C and ^{1}H–^{15}N heteronuclear multiple bond coherence (HMBC)) spectra were recorded on a JNM-ECA 500 NMR spectrometer (JEOL, Tokyo, Japan). Electrospray ionization (ESI) MS was carried out with a Platform LC mass spectrometer (Micromass MS Technologies, Manchester, UK). Samples for ESI-MS analysis were dissolved in methanol. IR and UV/VIS spectra were obtained using a Spectrum One FT-IR spectrometer (Perkin Elmer, Waltham, MA, USA) and a V-520 UV/VIS spectrophotometer (JASCO, Tokyo, Japan), respectively. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was carried out with a AXIMA-QIT mass spectrometer (Shimadzu, Kyoto, Japan) using 2,5-dihydroxybenzoic acid as an ionization matrix. RIF-6′-*N*-oxide (RIF-NO) was synthesized by the oxidation of RIF with *m*-chloroperbenzoic acid and its structure was confirmed by NMR analyses (Supplementary Tables S1–S3).

Construction of Δ*rox* mutant

The 3.1-kb *Sph*I–*Bam*HI fragment of pKNL027_N05 (Figure 1) was cloned into pK18*mobsacB*,[9] yielding pK*rox*. To make an in-frame deletion of *rox*, an inverse PCR with the primers rox5R (ATCGATCACGTCAGTCGTCCCAT) and rox3F (TGGTTCGGCGCCGCCACCTGA) was carried out, using pK*rox* DNA as the template. The amplified fragment was purified by agarose gel electrophoresis, its 5′ end was phosphorylated with T4 polynucleotide kinase (TOYOBO), and then the fragment was self-ligated, obtaining a deletion allele delivery plasmid pKΔ*rox*. Deletion mutants were selected as described in a previous paper.[2] Legitimate single and double crossover clones were detected by colony-direct PCR analyses[10] with appropriate combinations of the primers rox5F (ATC GGAACGACTGCCTCGGCACGGA), rox3R (AACTGGACGCCCATGTCGTC ACCGT), M13-325 (GTGCTGCAAGGCGATTAAGTTGG) and M13-RV507 (TCCGGCTCGTATGTTGTGTGGA). To complement the mutation, the 3.1-kb *Sph*I–*Bam*HI fragment of pKNL027_N05 was cloned into pNV118, yielding pNV118*rox*.

Decolorization of RIF by *Nocardia* strains

Nocardia strains were grown in 50 ml of BHI broth for 24 h at 37 °C. Cells were washed twice with sterile water, suspended in 50 ml of MM containing 50 µg ml^{-1} of RIF and incubated for 24 h at 37 °C. A total of 1 ml portions of cultures were taken and extracted with an equal volume of methanol. After removing cell debris by centrifugation, the extracts were subjected to reverse-phase HPLC using a Prominence system (Shimadzu) with a COSMOSIL $5C_{18}$-AR-II column (4.6 by 150 mm; Nacalai Tesque). The mobile phase consisted of 25 mM NaH_2PO_4:CH_3CN (65:35, v/v), running at a flow rate of 1 ml min^{-1}.

Phylogenetic analysis

Phylogenetic analysis was conducted using the MEGA program version 4.[11]

RESULTS

Identification of monooxygenated RIF

To demonstrate the RIF conversion activity of the protein encoded by *rox*, an expression plasmid pUC18*rox* was constructed. After various expression conditions were tested, it turned out that *E. coli* BL21(DE3)/pUC18*rox* grown in LB broth at 27 °C without the addition of IPTG yielded reproducible results. A total of 0.5 g of RIF was converted using resting cells of *E. coli* BL21(DE3)/pUC18*rox* and the reaction products were analyzed by TLC (Figure 2a). After

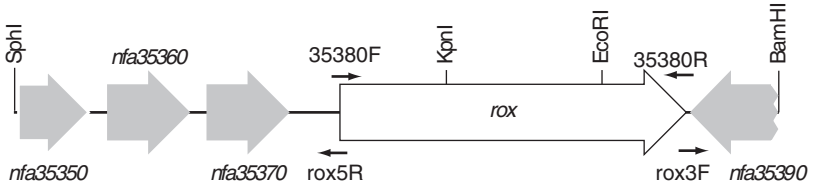

Figure 1 Map of the 3.1-kb *Sph*I–*Bam*HI fragment. The fragment was used for making a deletion allele and its complementation. Small arrows indicate primers used for cloning into pUC18 (35380F and 35380R) or inverse PCR (rox5R and rox3F), respectively.

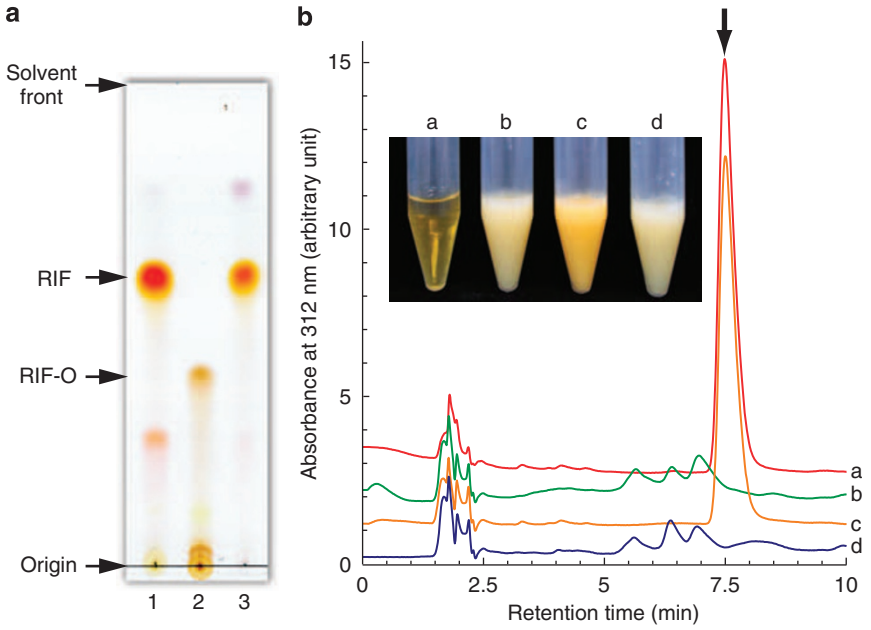

Figure 2 Conversion of rifampicin (RIF) by Rox. (**a**) RIF was incubated in 100 mM potassium phosphate buffer (pH 7.0) containing 1% glucose with *Escherichia coli* BL21(DE3) carrying pUC18 (lane 1) or pUC18*rox* (lane 2) or without *E. coli* (lane 3). Reaction mixtures were extracted with 50% methanol and subjected to thin layer chromatography (TLC). (**b**) RIF (50 μg ml^{-1}) was incubated with or without *Nicordia farcinica* strain in minimal media (MM) at 37 °C. The broths were subjected to high-performance liquid chromatography (HPLC) analysis or photographed after 24 and 72 h, respectively. a, no bacteria; b, wild-type; c, Δ*rox*; and d, Δ*rox*/pNV118*rox*. The arrow indicated RIF peak.

Table 1 NMR data for RIF and RIF derivatives in DMSO-d_6 (δ (ppm), *J* (Hz))[a]

	RIF			*RIF-O*			*RIF-NO*			*RIF-Om*
Position	δ_C	δ_H	δ_N	δ_C	δ_H	δ_N	δ_C	δ_H	δ_N	δ_N
4	145.6 s	12.50 s		184.6 s			145.2 s	12.45 s		
34	9.2 q	−0.27 d (6.7)		10.3 q	0.69 s (6.8)		9.0 q	−0.27 d (6.5)		
1′	137.8 d	8.08 s		155.0 d	8.28 s		136.1 d	8.04 s		
4′	47.9 t	3.38 m		53.7 t	3.08 t-like (4.7)		44.7 t, 45.4 t	3.40 m, 3.28 m		
5′	51.4 t	3.38 m		53.8 t	2.52 m		62.2 t, 62.7 t	2.94 m, 3.42 m, 3.37 m, 3.20 m		
7′	42.3 q	2.78 s		45.1 q	2.24 s		48.6 q, 58.9 q	3.15 s, 3.04 s		
2'			344.9			ND			347.5	321.2
3'			111.6			105.3			111.9	115.6
6'			38.1			31.1			103.7	ND

Abbreviations: ND, not detected; RIF, rifampicin.
[a]Only the data which were significantly different from RIF were shown. The numbers in parentheses denote coupling constants (*J*).

incubation for 24 h, the RIF spot (Rf 0.6) disappeared, whereas a spot with an R_F value of 0.4 was detected instead. This newly detected compound was extracted from the TLC plate and subjected to MALDI-TOF MS analysis. The compound showed a molecular ion peak $[M+Na]^+$ at *m/z* 861.46. Because this result indicates the addition of one oxygen atom to RIF ($[M+Na]^+$ at *m/z* 845.46), the compound was considered to be monooxygenated RIF (RIF-O). Therefore, we further purified the compound.

Structure elucidation of RIF-O

The molecular formula of RIF-O, $C_{43}H_{58}N_4O_{13}$, was confirmed by HRESI-MS (measured *m/z* 839.4083, calc for $C_{43}H_{59}N_4O_{13}$ $[M+H]^+$, 839.4079, Δ+0.4 mmu), indicating the addition of one oxygen atom to RIF.

The chemical shift assignment for ^{1}H- and ^{13}C-NMR of RIF-O and RIF was performed by the analysis of NMR experiments (DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC) (Table 1). In RIF-O, all the protons attached to the carbons, including the carbons in the ansa-chain (C15–29-O-C12) and in the piperazine moiety, were assigned successively to the same positions as RIF. The NH proton signals split into two peaks at 7.06 (0.5H) and 7.23 (0.5H), but both signals correlated to the amide-NH in ^{1}H–^{15}N HMBC experiments. These observations indicate that neither a change in the ansa-chain nor the addition of oxygen atom to any carbons occurred during the enzymatic conversion of RIF. On the other hand, the chemical shift of the H_3-34 signal (δ_H 0.69) of RIF-O was remarkably lower than that of RIF (δ_H −0.27). This implies the oxidation of the naphthoquinol moiety of RIF, because the same downfield shift has been reported in the case of oxidation of rifamycin SV (naphthoquinol form) to rifamycin S (naphthoquinone form).[12,13] This presumption was also supported by the lack of absorption at 470 nm in RIF-O (Supplementary Table S4). In IR spectra, RIF-O showed an additional peak at 1615 cm^{-1}, indicating the existence of an

additional carbonyl group in RIF-O. Furthermore, the C-4 signal (δ_C 184.6) of RIF-O suggested the existence of a quinine-like quaternary carbon (that is, quinone carbonyl).

Next, we tried to synthesize RIF-O by chemical oxidization of RIF with *m*-chloroperbenzoic acid but yielded RIF-6′-*N*-oxide (RIF-NO), an RIF metabolite with the same molecular formula as RIF-O.[14] Subsequently, we compared the NMR spectra of RIF-NO with those of RIF. The ^{15}N chemical shift (δ_N) was measured by ^{1}H–^{15}N HMBC (Table 1). The results showed that the chemical shifts at C-5′ and -7′ (α position of N-6′) moved to a lower magnetic field (Table 1). In addition, the chemical shift at N-6′ moved to a higher magnetic field (RIF, δ_N 38.1; RIF-NO, δ_N 103.7). These observations indicate that *N*-oxygenation of RIF resulted in chemical shift changes at the nitrogen, flanking protons and carbons. In the case of RIF-O, the signal changes were as follows. No large chemical shift changes were observed at N-3′ and N-6′ (Table 1 and Supplementary Table S3). Although the chemical shift at N-2′ of RIF-O could not be obtained, but that of a minor component (RIF-Om) was observed (δ_N 321.2). The chemical shift at N-2′ of RIF-Om was distinct from that of RIF. RIF-Om was always contained in the RIF-O preparation at a very low concentration (data not shown) and considered to be a tautomer of RIF-O. The downfield shifts of ^{13}C-NMR data at C-4′, -5′ and -7′ were minor. In contrast, a remarkable downfield shift was observed at C-1′ (RIF-O, δ_C 155.0; RIF, δ_C 137.8). These suggest a change at N-2′ in RIF-O as shown at N-6′ in RIF-NO.

Taken together all the above observations, we conclude that the structure of RIF-O is 2′-*N*-hydroxy-4-oxo-RIF (Figure 3a), and propose a mechanism involving the enzymatic addition of oxygen to the N-2′ of RIF, resulting in RIF-2′-*N*-oxide (RIF-Om), followed by RIF-O (Figure 3b).

Antimicrobial activity of RIF-O

The antimicrobial activity of RIF-O was evaluated (Table 2). The MIC values of RIF-O increased by more than 100-fold, thereby indicating a

Table 2 Susceptibility to RIF or RIF-O

	MIC ($\mu g\,ml^{-1}$)	
Organism	*RIF*	*RIF-O*
Escherichia coli NIHJ	16	>256
Klebsiella pneumoniae ATCC 10031	16	>256
Pseudomonas aeruginosa IFO 12582	32	>256
Kocuria rhizophila ATCC 9341	<0.125	32
Staphylococcus aureus ATCC 6538P	<0.125	16
Bacillus subtilis PCI 219	<0.125	64
Mycobacterium smegmatis IFM 0813	32	>256
Nocardia farcinica IFM 10152 (wild-type)	512	
N. farcinica IFM 10152 Δ*rox*	512	
N. farcinica IFM 10152 Δ*rox*/pNV118*rox*	512	
N. farcinica IFM 10152 Δ*rpoB2*	0.25	
N. farcinica IFM 10152 Δ*rpoB2*/pNV118*rox*	8	

Abbreviations: MIC, minimum inhibitory concentration; RIF, rifampicin.

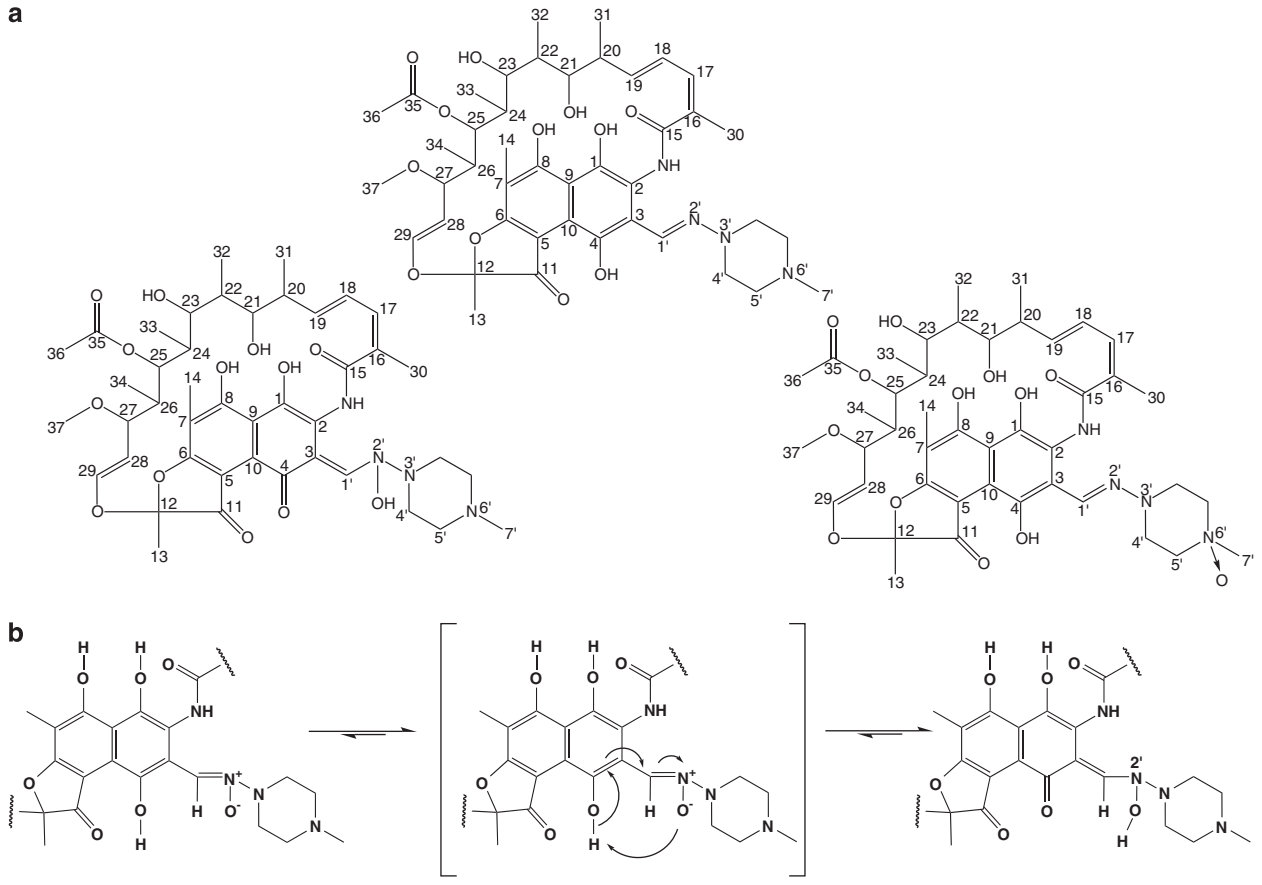

Figure 3 (**a**) Structures of rifampicin (RIF) (middle), RIF-O (left) and RIF-NO (right). (**b**) Proposed scheme of monooxygenation. Oxygen addition to the N-2′ of RIF results in RIF-2′-*N*-oxide (RIF-Om) (left), followed by RIF-O (right).

more than 100-fold reduction in antimicrobial activity. However, RIF-O retained its antimicrobial activity against *K. rhizophila*, *S. aureus* and *B. subtilis*. This suggests that RIF was not completely inactivated by Rox protein.

RIF resistance conferred by *rox*

In order to elucidate the role of *rox* in the RIF resistance of *N. farcinica*, we constructed an in-frame unmarked deletion mutant, Δ*rox*, as described in MATERIALS AND METHODS. The MIC value of a Δ*rox* strain was the same as that of the wild-type strain (Table 2). To complement the mutation, we cloned a *rox*-containing DNA fragment (Figure 1) into pNV118. The resultant plasmid pNV118*rox* was introduced into the Δ*rox* strain. However, no significant change in the MIC value was observed after the introduction. We next transformed the Δ*rpoB2* strain[2] with pNV118*rox*. The Δ*rpoB2* strain was susceptible to 0.25 μg ml^{-1} of RIF, whereas the strain carrying pNV118*rox* showed elevated resistance to RIF with an MIC of 8 μg ml^{-1} (a 32-fold increase).

Role of *rox* in RIF decolorization

We investigated the RIF-converting ability of the Δ*rox* strain. When RIF was incubated with the wild-type *N. farcinica* strain, neither RIF

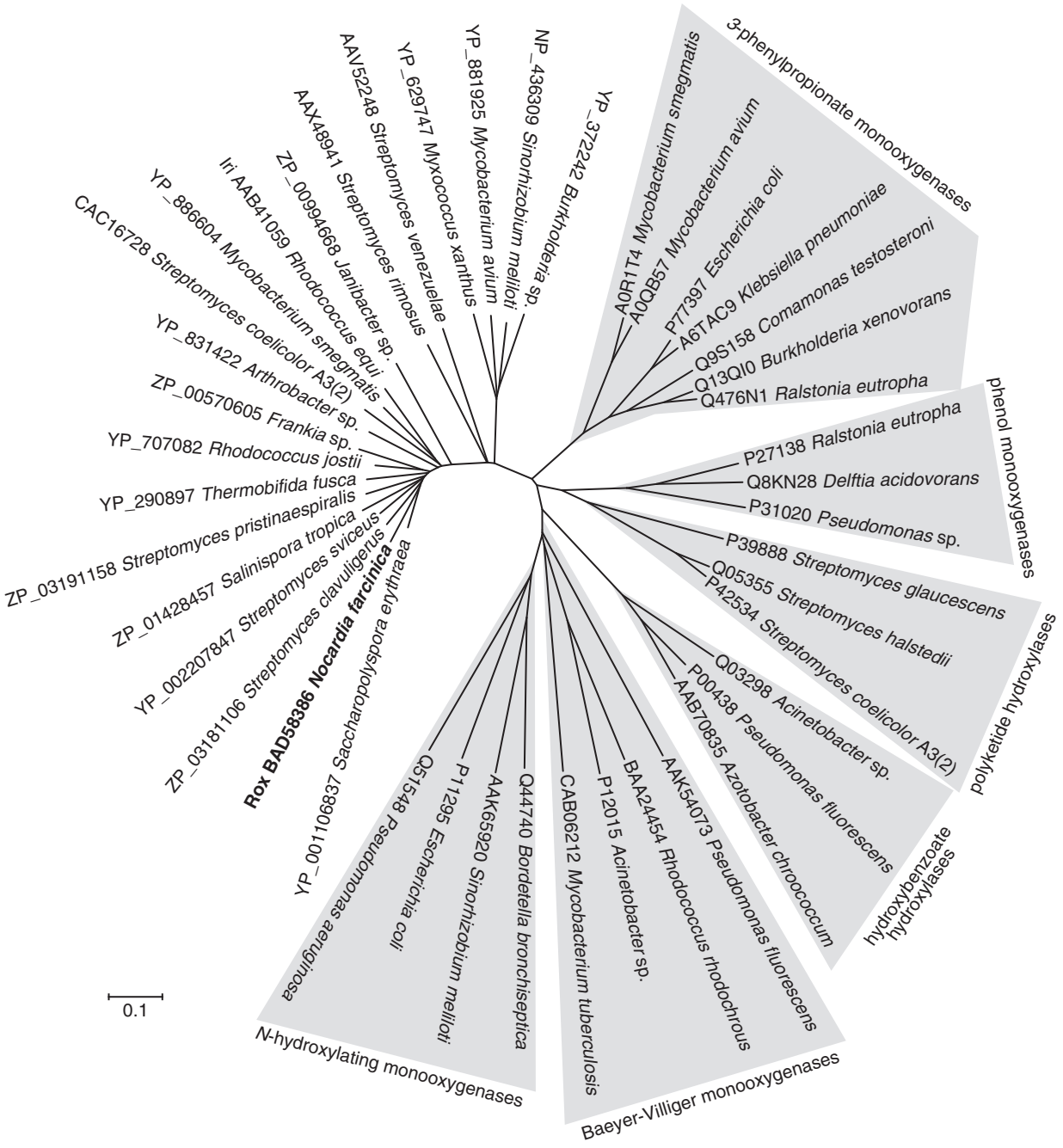

Figure 4 Phylogenetic analysis of known flavin adenine dinucleotide (FAD)-dependent monooxygenases and proteins highly homologous to Rox. An unrooted tree was obtained by the neighbor-joining method with multiple alignment of protein sequence using the MEGA program.[10] Shaded areas denote protein families, which include functionally similar known FAD-dependent monooxygenases.

nor its color was detectable after 24 h and after 72 h, respectively (Figure 2b), suggesting the degradation of RIF. In contrast, RIF seemed to be intact in the Δ*rox* strain after incubation for 24 h. Furthermore, the loss of ability of the Δ*rox* strain to degrade RIF was completely restored by transformation with pNV118*rox*. These results suggest that monooxygenation by Rox protein is required to decolorize RIF.

DISCUSSION

As described above, we demonstrated that the *rox* gene encodes an RIF monooxygenase. Monooxygenases constitute a large, divergent protein family and many of them are flavin adenine dinucleotide (FAD)-dependent enzymes. Rox protein is also considered to be a FAD-dependent monooxygenase because it contains the FAD-binding domain (Pfam accession number PF01494). Although the determination of the RIF-O structure demonstrated that Rox protein was a monooxygenase adding an oxygen to the N-2′ position of RIF (Figure 3), Rox protein seems to be different from known FAD-dependent monooxygenases. Phylogenetic analysis of known FAD-dependent monooxygenases and proteins highly homologous to Rox clearly showed that the Rox-containing protein family is distinguishable from those consisting of known FAD-dependent monooxygenases (Figure 4). Little is known about the functions of the Rox-containing family proteins and, therefore, we believe Rox should be the first enzyme in this family for which the real reaction has been experimentally determined. From this point of view, it would be interesting to know the detailed reaction mechanism and structure of this enzyme.

Rifampicin was converted to a reddish compound (RIF-O) by *E. coli* carrying the *rox* gene, but was completely decolorized by the wild-type *N. farcinica* strain (Figure 2b). This indicates the involvement of additional steps in the decolorization of RIF by *N. farcinica*. Furthermore, the inability of the Δ*rox* strain to decolorize RIF suggests that decolorization is probably initiated by monooxygenation by Rox protein. Further study will be required to determine how RIF is decolorized after monooxygenation.

No difference in RIF resistance was observed between the wild-type and Δ*rox* strains (Table 2). This indicates that the *rpoB2* gene is the principal RIF resistance determinant of *N. farcinica*.[2] However, a partial restoration of RIF resistance in the Δ*rpoB2* strain carrying pNV118*rox* suggests that *rox* plays a role as the secondary RIF-resistance factor in *N. farcinica*. It is considered that the Δ*rpoB2* strain, especially the pNV118*rox*-carrying strain, was able to convert a relatively low amount of RIF using the Rox protein produced before the addition of RIF and then grew as the RIF concentration decreased. These observations led us to a notion that *rpoB2* and *rox* may have roles like vanguard and rear-guard, respectively, to resist RIF in this microorganism.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)