ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 2006, p. 1342–1346 Vol. 50, No. 4
0066-4804/06/$08.00+0 doi:10.1128/AAC.50.4.1342–1346.2006

Contribution of *rpoB2* RNA Polymerase β Subunit Gene to Rifampin Resistance in *Nocardia* Species

Jun Ishikawa,[1]* Kazuhiro Chiba,[1,2] Haruyo Kurita,[1] and Hiroyuki Satoh[2]

Department of Bioactive Molecules, National Institute of Infectious Diseases, Tokyo,[1] and Department of Biomolecular Science, Faculty of Science, Toho University, Chiba,[2] Japan

Received 29 November 2005/Returned for modification 27 December 2005/Accepted 16 January 2006

Nocardia **species are gram-positive environmental saprophytes, but some cause the infectious disease nocardiosis. The complete genomic sequence of *Nocardia farcinica* IFM 10152 has been determined, and analyses indicated the presence of two different RNA polymerase β subunit genes, *rpoB* and *rpoB2*, in the genome (J. Ishikawa, A. Yamashita, Y. Mikami, Y. Hoshino, H. Kurita, K. Hotta, T. Shiba, and M. Hattori, Proc. Natl. Acad. Sci. USA 101:14925–14930, 2004). These genes share 88.8% identity at the nucleotide level. Moreover, comparison of their amino acid sequences with those of other bacterial RpoB proteins suggested that the nocardial RpoB protein is likely to be rifampin (RIF) sensitive, whereas RpoB2 protein contains substitutions at the RIF-binding region that are likely to confer RIF resistance. Southern analysis indicated that *rpoB* duplication is widespread in *Nocardia* species and is correlated with the RIF-resistant phenotype. The introduction of *rpoB2* by using a newly developed *Nocardia-Escherichia coli* shuttle plasmid vector and transformation system conferred RIF resistance to *Nocardia asteroides* IFM 0319^T, which has neither RIF resistance nor *rpoB* duplication. Furthermore, unmarked *rpoB2* deletion mutants of *N. farcinica* IFM 10152 showed no significant resistance to RIF. These results indicated the contribution of *rpoB2* to RIF resistance in *Nocardia* species. Since this is the first example of genetic engineering of the *Nocardia* genome, we believe that this study, as well as our determination of the *N. farcinica* genome sequence, will be a landmark in *Nocardia* genetics.**

Nocardia species are gram-positive environmental saprophytes, but some cause the infectious disease nocardiosis. *Nocardia farcinica* is thought to be the most important species from the viewpoint of multiple drug resistance and due to its recent isolation incidence in Japan (8) as well as in Europe (14, 20). Recently we determined the complete genomic sequence of a clinical isolate, *N. farcinica* IFM 10152, and deduced the molecular bases of virulence and multidrug resistance of this bacterium (7). Unexpectedly, the genome contained two different RNA polymerase (RNAP) β subunit genes, *rpoB* and *rpoB2*. *rpoB* is considered to be the major RNAP β subunit gene because it forms an operon with *rpoC*, encoding the RNAP β′ subunit, as is the case in many bacteria. On the other hand, *rpoB2* is located 570 kb from *rpoB* and with no other RNAP subunit genes. *rpoB* and *rpoB2* share 88.8% identity at the nucleotide level. Moreover, comparison of their amino acid sequences with those of other bacterial RpoB proteins suggests that the nocardial RpoB protein is likely to be sensitive to rifampin (RIF), whereas the RpoB2 protein contains substitutions at positions which result in RIF resistance in many bacteria (16). Based on these observations, we predicted that *rpoB2* was capable of contributing to RIF resistance of *N. farcinica* IFM 10152 (7).

RIF remains a front-line drug for treatment of infectious diseases, especially tuberculosis. Resistance to RIF in clinical isolates is almost always due to point mutations in the *rpoB* gene. In contrast, *Nocardia* and related bacteria intrinsically possess a variety of RIF-inactivating enzymes (1, 12, 22, 23). However, there have been no reports of RIF resistance involving *rpoB* duplication, implying a novel resistance mechanism. To elucidate the contribution of *rpoB2* to RIF resistance, we constructed an *rpoB2* deletion mutant of *N. farcinica* IFM 10152 and introduced the wild-type *rpoB2* into *Nocardia asteroides* IFM 0319^T, which was sensitive to RIF and lacked *rpoB* duplication, using a newly developed *Nocardia-Escherichia coli* shuttle plasmid vector and transformation system.

* Corresponding author. Mailing address: Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku, Tokyo 162-8640, Japan. Phone: 81 3 5285 1111. Fax: 81 3 5285 1272. E-mail: jun@nih.go.jp.

MATERIALS AND METHODS

Bacterial strains and plasmids. All *Nocardia* strains were obtained from the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan. With the exception of type strains, all *Nocardia* strains were isolated from clinical specimens in Japan. For cloning experiments, *E. coli* JM109 was used as a host strain. pYUB12 (18) was provided by S. K. Das Gupta and used as the source of a 2.6-kb EcoRV-HpaI fragment containing the pAL5000 origin of replication (17). pIJ702 was used as the source of a 1.05-kb BclI fragment carrying the *tsr* gene of *Streptomyces azureus* (3). A 6.7-kb SphI fragment carrying *rpoB2* was prepared from pKNL039_H03, a plasmid from the *N. farcinica* IFM 10152 ordered plasmid library (http://nocardia.nih.go.jp/). pK18*mobsacB* (15) was obtained from the National Institute of Genetics, Japan.

RIF resistance test. *Nocardia* strains were incubated in brain heart infusion (BHI) broth (BD Biosciences) at 37°C for 24 h. A loopful of the culture was streaked onto BHI agar plates containing 0, 5, 10, 25, 50, or 100 μg/ml of RIF. The plates were incubated at 37°C, and bacterial growth was scored after 24 and 48 h.

Transformation of *Nocardia*. Nocardia strains were incubated in 10 ml of BHI broth for 18 to 24 h at 37°C. Cells were harvested, washed twice with 5 ml of ice-cold water, and then resuspended in 50 μl of ice-cold 10% glycerol. The suspension was transferred to a chilled electroporation cuvette (2-mm gap) and mixed with 0.3 to 0.5 μg of DNA. After pulsing at 12.25 kV/cm with an Electro Cell Porator 600 (BTX Inc.), the suspension was added to 900 μl of BHI broth and incubated for 2 h at 37°C. Cells were then plated onto BHI agar plates containing 50 μg/ml of thiostrepton or 25 μg/ml of neomycin and incubated for 2 to 3 days at 37°C.

DNA techniques. For nucleotide sequencing, BigDye Terminator v3.1 cycle sequencing kits and a 3130 Genetic Analyzer (Applied Biosystems) were used in accordance with the manufacturer's instructions. For Southern hybridization, the

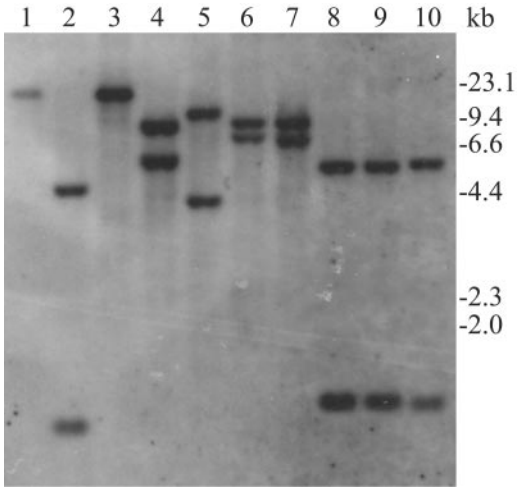

FIG. 1. Distribution of *rpoB* duplication among *Nocardia* strains. Total DNAs extracted from *N. asteroides* IFM 0319^T (lane 1), *N. asteroides* IFM 10159 (lane 2), *N. asteroides* IFM 10162 (lane 3), *N. brasiliensis* IFM 0236^T (lane 4), *N. brasiliensis* IFM 0406 (lane 5), *N. brasiliensis* IFM 10132 (lane 6), *N. brasiliensis* IFM 10160 (lane 7), *N. farcinica* IFM 0284^T (lane 8), *N. farcinica* IFM 10125 (lane 9), and *N. farcinica* IFM 10152 (lane 10) were digested with BamHI and probed with a 437-bp fragment containing the C-terminal region of *rpoB*. The probe was prepared from the total DNA of *N. farcinica* IFM 10152 by PCR using the primers NFrpoBF and NFrpoBR.

AlkPhos direct labeling and detection system (Amersham Biosciences) was used. PCR was carried out in 10-μl reaction mixtures with a KOD-Plus kit (TOYOBO). For screening of transformants by PCR, bacterial cells were used as the template (6). 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 58 or 60°C, and 20 s at 68°C, with a final extension step at 68°C for 5 min. The following PCR primers were used: rpoBcoreF, 5′-CCGCAGACCCTGATCAACATCC-3′; rpoBcoreR, 5′-TCATGCTCGAGGAACGGAATCATC-3′; NFrpoBF, 5′-ATCGGCCAGATCCTGGAAACCCAC-3′; NFrpoBR, 5′-CATCGCCCAGCACTCCATCTCAC-3′; rpoBWF, 5′-GACGTCGACAAGCGCGACACC-3′; rpoBWR, 5′-GATGATCGCGTCCTCGTAGTTGTG-3′; aph3IIaF, 5′-TGCTCCTGCCGAGAAAGTAT-3′; and aph3IIaR, 5′-AATATCACGGGTAGCCAACG-3′.

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the DDBJ database under accession numbers AB219431, AB243741, and AB243742.

RESULTS

Correlation between *rpoB* duplication and RIF resistance in *Nocardia* species. To analyze the occurrence of *rpoB* duplication in *Nocardia* species, total DNAs were extracted from 10 strains (including 3 type strains), digested with BamHI, and probed with a 437-bp fragment containing the C-terminal region of *rpoB* (Fig. 1). Since the nucleotide sequence of this region is identical to that of the corresponding region of *rpoB2*, two bands were expected to be detected in *N. farcinica* IFM 10152. The results showed that two bands were detected in 8 of 10 strains, suggesting the occurrence of *rpoB* duplication in these 8 strains. In contrast, single bands were obtained from *N. asteroides* IFM 0319^T and IFM 10162, suggesting a lack of *rpoB* duplication. We also probed KpnI-digested total DNAs with the same fragment, with equivalent results (data not shown).

Next we examined the RIF resistance of 10 strains. All strains with two bands were resistant to RIF (>25 μg/ml) with the exception of *Nocardia brasiliensis* IFM 0406 (Table 1). These observations suggested a correlation between *rpoB* duplication and RIF resistance.

Sequence analysis of the RIF-binding region of RpoB. The above results strongly suggested that *rpoB* duplication is involved in RIF resistance in *Nocardia* species. This possibility could be confirmed by the introduction of *rpoB2* into a strain with neither *rpoB* duplication nor RIF resistance. Southern hybridization analysis indicated that *N. asteroides* IFM 0319^T is likely to have only one copy of *rpoB* in its genome (Fig. 1, lane 1). Subsequently, to estimate sensitivity to RIF, the RIF-binding region of *rpoB* was amplified from the IFM 0319^T genome by PCR using the primers rpoBcoreF and rpoBcoreR, and its nucleotide sequence was determined. The deduced amino acid sequence of the *rpoB* gene of IFM 0319^T was identical not only to that of IFM 10152 but also to that of *Mycobacterium tuberculosis* H37Rv, which has been shown to be sensitive to RIF (Fig. 2). Therefore, RpoB of IFM 0319^T is considered to be sensitive to RIF.

We also amplified the RIF-binding regions of *rpoB* and *rpoB2* from the *N. farcinica* IFM 0284^T genome by PCR using the primers rpoBWF and rpoBWR and determined their nucleotide sequences. Although a few nucleotide sequence differences in both *rpoB* and *rpoB2* were found between IFM 10152 and IFM 0284, there were no deduced amino acid sequence differences between them (data not shown).

Introduction of *rpoB2* into *N. asteroides* IFM 0319^T. Since very few vectors for use in *Nocardia* have been reported (21), we constructed a new *Nocardia-E. coli* shuttle plasmid vector employing pAL5000 (10), which is the most frequently used plasmid for making mycobacterial vectors. A newly developed vector, pNV1.2, was constructed by inserting a 2.6-kb EcoRV-HpaI fragment carrying the pAL5000 origin of replication (2)

TABLE 1. *Nocardia* strains and their RIF resistance

Species and strain/plasmid	RIF resistance (μg/ml) at:		Source
	24 h	48 h	
N. asteroides			
IFM 0319^T	<5	<5	Type strain (ATCC 19247^T)
IFM 10159	<5	25	Japan clinical isolate
IFM 10162	<5	<5	Japan clinical isolate
N. brasiliensis			
IFM 0236^T	10	50	Type strain (ATCC 19296^T)
IFM 0406	<5	<5	Japan clinical isolate
IFM 10132	>100	>100	Japan clinical isolate
IFM 10160	10	50	Japan clinical isolate
N. farcinica			
IFM 0284^T	>100	>100	Type strain (ATCC 3318^T)
IFM 10125	25	>100	Japan clinical isolate
IFM 10152	>100	>100	Japan clinical isolate
IFM 10152 Δ*rpoB2*	<5	<5	This study
IFM 10152 Δ*rpoB2*/pNV*rpoB2*	>100	>100	This study
N. asteroides			
IFM 0319^T/pNV*rpoB2*	50	>100	This study
IFM 0319^T/pNV1.2	<5	<5	This study

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                                  Cluster I
RpoB   VAAIKEFFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGL
RpoB2  VAAMKEFFGTSQLSQFMDQRNPLASLTNKRRLSALGPGGL
Nast   VAAIKEFFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGL
Mtub   VAAIKEFFGTSQLSQFMDQNNPLSGLTHKRRLSALGPGGL

                                            Cluster II
RpoB   SRERAGLEVRDVHPSHYGRMCPIETPEGPNIGLIGSLSVY
RpoB2  SRERAGLEVRDVHYSHYGRMCPIETPEGPNIGLMGYLSVY
Nast   SRERAGLEVRDVHPSHYGRMCPIETPEGPNIGLIGSLSVY
Mtub   SRERAGLEVRDVHPSHYGRMCPIETPEGPNIGLIGSLSVY
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FIG. 2. Alignment of the RIF-binding regions of RNAP β subunits among *N. farcinica* IFM 10152 (RpoB and RpoB2), *N. asteroides* IFM 0319^T (Nast), and *M. tuberculosis* H37Rv (Mtub). Amino acid substitutions are represented in reverse color. RIF-binding regions (clusters I and II) are boxed.

and a 1.05-kb BclI fragment containing the thiostrepton resistance gene (*tsr*) of *Streptomyces azureus* (3) into the HincII and BamHI sites of pUC18, respectively (Fig. 3). Next, a 6.7-kb SphI fragment containing *rpoB2* was inserted into pNV1.2, resulting in pNV*rpoB2* (Fig. 3).

N. asteroides IFM 0319^T was transformed with pNV*rpoB2* or pNV1.2 by electroporation. Transformants carrying pNV*rpoB2* were able to grow in the presence of RIF (100 μg/ml), but control strains carrying pNV1.2 were not (Table 1). This result indicated that *rpoB2* acts as a determinant of RIF resistance in *N. asteroides*.

Construction of *rpoB2* deletion mutants and their RIF resistance. To determine the role of *rpoB2* in RIF resistance in *N. farcinica* IFM 10152, we constructed an in-frame, unmarked deletion of *rpoB2* by using a two-step selection method (Fig. 4A). A 4.9-kb ScaI-SphI fragment containing the wild-type *rpoB2* was ligated to pUC19 that was digested with HincII and SphI. To make an in-frame deletion, the internal 2.9-kb StuI fragment of the wild-type allele was deleted by digestion of the resulting plasmid with StuI followed by self-ligation. A 2.0-kb XbaI-HindIII fragment carrying a deletion allele was cloned into pK18*mobsacB* (15), yielding pNVD*rpoB2*Δ*StuI*. After electroporation of pNVD*rpoB2*Δ*StuI* into IFM 10152, 95 neomycin-resistant clones were obtained. Of these, seven appeared to be legitimate single-crossover recombinants, which were distinguished from the rest by sensitivity to 10% sucrose and by the results of PCR analyses. One of the seven recombinants was grown in the presence of 10% sucrose, and sucrose-resistant and neomycin-sensitive clones were obtained. Six of the seven clones analyzed were confirmed to possess the expected genotype by Southern hybridization (Fig. 4B). To avoid cross-hybridization between *rpoB* and *rpoB2*, a 0.6-kb EcoRI fragment which contained the *nfa46450* gene flanking *rpoB2* was prepared from pNVD*rpoB2*Δ*StuI* and used as a probe (Fig. 4A). In Δ*rpoB2* mutants, the 6.6-kb NotI fragment had disappeared and a 3.7-kb NotI fragment was detected instead, reflecting loss of the 2.9-kb StuI fragment (Fig. 4B).

The wild-type strain was resistant to RIF at the lowest level of 100 μg/ml, whereas the Δ*rpoB2* mutant was unable to grow in the presence of 5 μg/ml RIF (Table 1). In contrast, a Δ*rpoB2* mutant carrying pNV*rpoB2*, which contained an intact *rpoB2* gene (Fig. 3), recovered RIF resistance (Table 1). This result would rule out that the sensitivity of the Δ*rpoB2* mutant to RIF was due to a mutation other than the deletion of *rpoB2*.

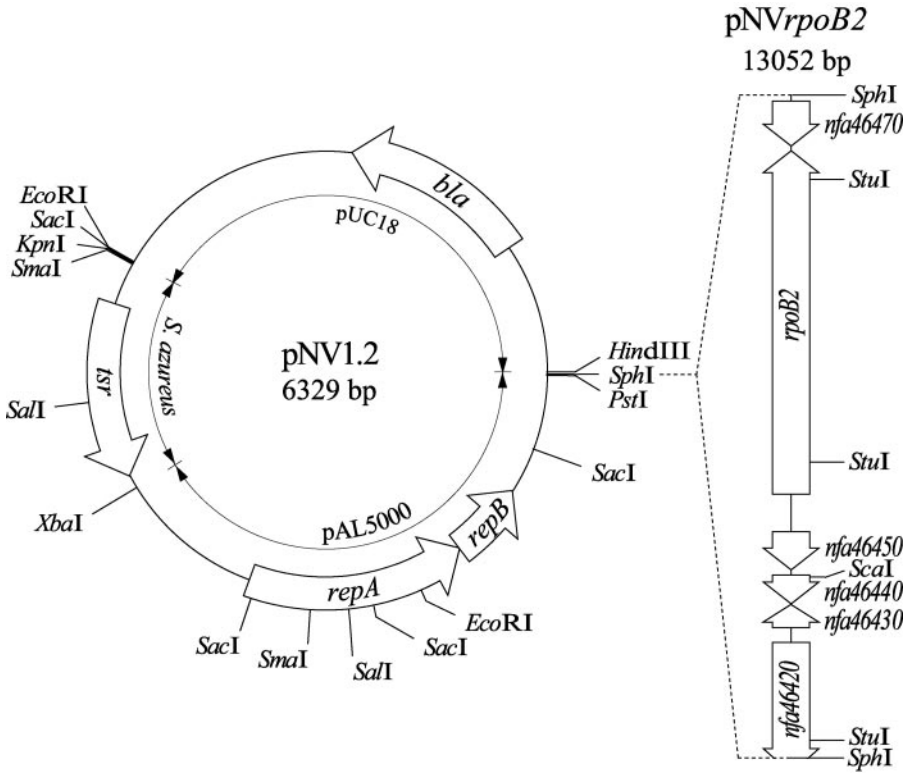

FIG. 3. Restriction maps of pNV1.2 and pNV*rpoB2*. See text for details.

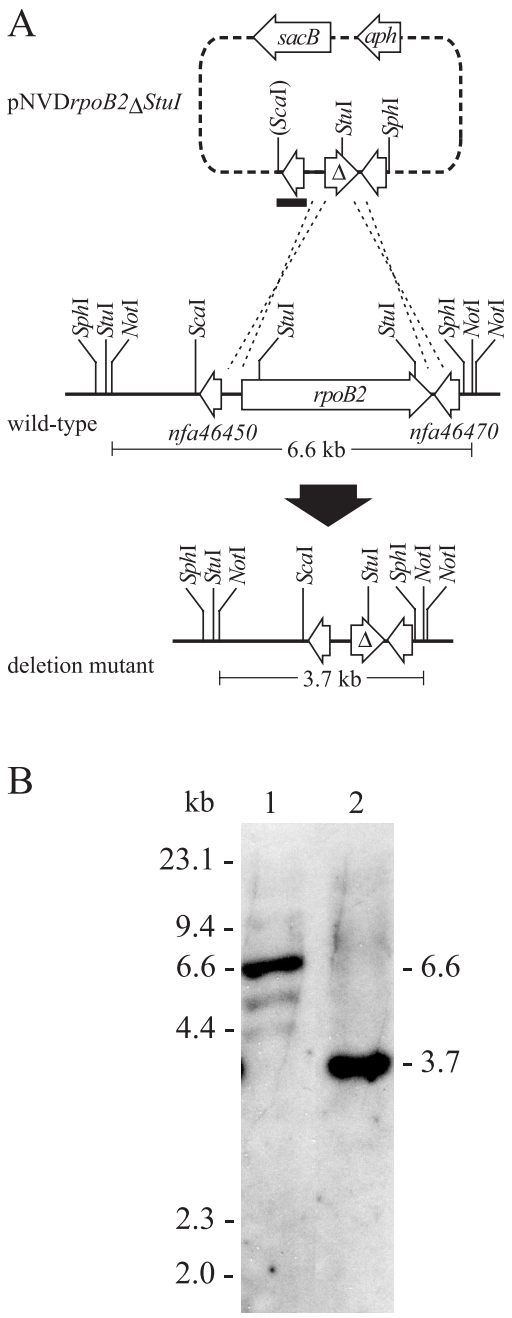

FIG. 4. Construction of the Δ*rpoB2* mutant. A. Strategy for making an in-frame, unmarked deletion of *rpoB2*. See text for details. Δ indicates a deletion allele. The ScaI half-site generated after ligation of a 4.9-kb ScaI-SphI fragment containing *rpoB2* to pUC19 digested with HincII and SphI is shown in parentheses. B. Southern hybridization analysis of a Δ*rpoB2* mutant. NotI-digested total DNAs extracted from the wild-type strain (lane 1) and a Δ*rpoB2* mutant (lane 2) were probed with a 0.6-kb EcoRI fragment containing *nfa46450* (short black bar in panel A).

DISCUSSION

We demonstrated here the contribution of *rpoB2* to RIF resistance in *Nocardia* species. There are at least two possible mechanisms by which *rpoB2* gives rise to RIF resistance. The first is that RpoB2 may have reduced binding affinity for RIF due to amino acid substitutions and is capable of functioning in the presence of RIF instead of RpoB. This is the most commonly observed type of RIF resistance mechanism in many bacteria and can be confirmed by investigating the direct interaction between RpoB2 and RIF. The acquisition of antibiotic resistance due to mutations which cause structural changes in the target of the drug is often disadvantageous to bacteria in the absence of antibiotics (2, 13). For example, RIF-resistant *rpoB* mutants of *M. tuberculosis* show a decreased growth rate in vitro and in macrophages (11). However, since such disadvantages can be minimized by carrying both the wild-type *rpoB* and a mutant *rpoB* (e.g., *rpoB2*) in one genome, this may be an elaborate strategy to withstand RIF.

The second possible mechanism is that RNAP with RpoB2 may elicit expression of a latent RIF resistance gene which may be present in the genome. RIF mutations in *rpoB* have been shown to affect gene expression. For example, mutations in *rpoB* produce increased or decreased expression of genes controlled by a stringent promoter (24), and certain RIF mutations in *rpoB* have been shown to result in elevated antibiotic production in *Streptomyces* (4) and *Bacillus subtilis* (5). On the other hand, a variety of RIF-inactivating enzymes have been identified in *Nocardia* and related taxa, such as enzymes involved in phosphorylation (23), glycosylation (22), ribosylation (12), and monooxygenation (1). Indeed, *N. farcinica* IFM 10152 possesses a monooxygenase gene (*nfa35380*) whose deduced amino acid sequence is highly homologous to that of the RIF monooxygenase of *Rhodococcus equi* (1). Further study will be required to confirm the involvement of *nfa35380* in the RIF resistance of *N. farcinica* IFM 10152. Yazawa et al. reported that *N. farcinica* strains probably inactivated RIF by decomposition (22). *N. farcinica* IFM 10152 also decolorizes RIF in prolonged culture (unpublished data), implying the participation of decomposition in RIF resistance. However, no growth was observed even when Δ*rpoB2* mutants were cultured for 1 week in the presence of 5 μg/ml RIF (data not shown). These observations may support the second possibility in *N. farcinica*.

N. asteroides IFM 10159 was resistant to RIF only after 48 h, and *N. brasiliensis* IFM 0406 was sensitive to RIF. These observations may be due to the weak expression of *rpoB2*. Our preliminary experiments showed that the expression of *rpoB2* was less than 1/10 that of *rpoB* (unpublished data). In IFM 0406, the expression of *rpoB2* may be too weak to contribute to the RIF resistance of the host.

Southern analysis indicated that *rpoB* duplication is widespread in *Nocardia* strains and species (Fig. 1). *rpoB* duplication may not be a rare event in *Nocardia* and related taxa, because *rpoB* duplication has recently been found in *Actinomadura* sp. strain ATCC 39727 (19), which is closely related to *Nocardia*. The extra *rpoB* gene of ATCC 39727, $rpoB^R$, was studied in connection with antibiotic production. The constitutive expression of $rpoB^R$ led to increased production of the glycopeptide antibiotic A40926 in a mutant resistant to RIF. In this context, it would be interesting to determine the effects of *rpoB2* expression on the antibiotic productivity of *N. asteroides* IFM 0319^T carrying pNV*rpoB2*.

Nocardia has interesting and important features because some species are known to produce antibiotics and aromatic

compound-degrading or -converting enzymes. However, the genetic manipulation of this organism has been hampered by the lack of genetic tools. We showed here that the mycobacterial plasmid pAL5000 was capable of replicating in *Nocardia* species. This would facilitate faster progress in the molecular biology of *Nocardia* because a number of mycobacterial vectors may be available, with or without slight modification. We also demonstrated that the *Nocardia* genome can be modified by standard techniques. Since this is the first example of genetic engineering of the *Nocardia* genome, we believe that this study, as well as our determination of the *N. farcinica* genome sequence, will be a landmark in *Nocardia* genetics. However, the frequency of homologous recombination-mediated integration events obtained using a suicide plasmid was found to be very low. Only 7 of 95 recombinants appeared to be legitimate single-crossover recombinants. The rest would be generated by illegitimate recombination, because the *aph* gene was detected in all neomycin-resistant recombinants by PCR with the primers aph3IIaF and aph3IIaR (data not shown). A high degree of illegitimate recombination has been known to occur in slow-growing mycobacteria (9). It is necessary to develop a more efficient strategy for gene knockout in *Nocardia*.

ACKNOWLEDGMENTS

We thank S. K. Das Gupta for pYUB12, Y. Mikami for *Nocardia* strains, K. Takeda and F. Saito for technical assistance, K. Ishino and Y. Hoshino for helpful discussion, K. Summers and N. Summers for help with the text, and K. Hotta and Y. Uehara for critical reading of the manuscript.

This work was supported in part by the Research for the Future Program of the Japan Society for the Promotion of Science (grant no. 00L01411) and by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture (grant no. 17019009).

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