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Original Article

Construction of a Pair of Practical *Nocardia-Escherichia coli* Shuttle Vectors

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SUMMARY: We constructed a pair of *Nocardia-Escherichia coli* shuttle vectors, pNV18 and pNV19, by combining the mycobacterial plasmid pAL5000 with the *E. coli* vector pK18 or pK19. These vectors have a number of useful features, including small size (4.4 kb), a multiple cloning site, and blue/white selection. To our knowledge, pNV18 and pNV19 are the first cloning vectors for practical use in *Nocardia* spp.

INTRODUCTION

Nocardia spp. are Gram-positive bacteria and are widely distributed in the natural environment, but some species are often isolated from clinical specimens as the causative agents of nocardiosis. *Nocardia* spp. also have industrial importance because of their ability to produce antibiotics and to catalyze bioconversion or biodegradation of chemicals. Recently, we determined the complete genome sequence of a clinical isolate *N. farcinica* IFM 10152 and deduced the molecular bases of virulence, multidrug resistance, and secondary metabolism in this organism (1).

Despite their versatility, genetic manipulation of *Nocardia* has been hampered by a lack of genetic tools. Although a cloning vector system for *Nocardia* has been developed (2), it has not been used extensively. Recently, we developed a transformation system for *Nocardia* and showed that the *Nocardia* genome can be engineered by standard molecular biological techniques, i.e., gene knockout based on homologous recombination (3). We also constructed the plasmid pNV1.2 carrying the origin of replication of the mycobacterial plasmid pAL5000 (4) and the *tsr* gene (5) as a selection marker in *Nocardia*. Although pNV1.2 can be used as a cloning vector in *Nocardia*, it is not suitable for general use, as it has neither a multiple cloning site nor secondary selection markers. Therefore, we constructed a pair of shuttle vectors for practical use.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions: All *Nocardia* strains were provided by the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan. For cloning experiments, *Escherichia coli* JM109 was used as the host strain. pYUB12 (6) carrying the entire sequence of pAL5000 (7) was provided by Prof. S. K. Das Gupta. pK18 and pK19 (8) were obtained from the National Institute of Genetics, Japan. All strains were cultured at 37°C in brain heart infusion (BHI) broth (BD Biosciences, Sparks, Md., USA) or on BHI agar plates.

PCR: A KOD -Plus- kit (Toyobo, Tokyo, Japan) was used for PCR. To obtain a 1,777-bp fragment containing the pAL5000 origin of replication, the primers NheIpAL5000N2 (CCTT GCTAGCGTGAGTAGCGCGTAC) and pAL5000CNheI (AGAAAGCTAGCGTCTACCAGGACTT) were used.

Site-directed mutagenesis: To remove the *Eco*RI and *Sal*I/*Hin*cII sites located in the pAL5000 origin of replication, a QuickChage II site-directed mutagenesis kit (Stratagene, La Jolla, Calif., USA) was used with the primers mpNV1EcoRI (CACGCGCGAAACGCCGAGTTCCCGTGCAACGACGT), mpNV2EcoRI (CACGTCGTTGCACGGGAACTCGGCGT TTCGCGCGT), mpNV1HincII (GGCCTTCGGCGCGCCG TGGACGGCGACCGCAGTTA), and mpNV2HincII (GTA ACTGCGGTCGCCGTCCACGGCGCGCCGAAGGC).

Transformation: *Nocardia* strains were transformed with 50 - 100 ng of DNA by the method reported previously (3).

Plasmid stability: Plasmid stability was determined according to the method reported by Gavigan et al. (9) with slight modifications. *N. farcinica* IFM 10152 carrying pNV19 was grown for 24 h in 10 ml of BHI broth containing 10 μg/ml of neomycin. Cultures were diluted 1:100 in antibiotic-free medium and grown for 24 h. This process was repeated daily for 4 days. Each culture was diluted appropriately and plated out on BHI agar plates with or without 25 μg/ml of neomycin. Colonies were scored after incubation for 2 days, and the stability of the plasmid was determined as the proportion of colonies resistant to neomycin compared with the total number of cells.

Resistance test: *Nocardia* strains were incubated in BHI broth for 24 h. A loop of the culture was streaked onto BHI agar plates containing 0, 5, 10, 25, 50, or 100 μg/ml of antibiotic. The plates were incubated at 37°C, and bacterial growth was scored after 48 h.

RESULTS

Construction of the shuttle vectors pNV18 and pNV19: A pair of shuttle vectors was constructed by combining the mycobacterial plasmid pAL5000 (10) with pK18 or pK19 (8). The pAL5000 origin of replication is known to be contained in a 2.6-kb *Eco*RV-*Hpa*I fragment (4). However, this fragment also contains dispensable sequences (11). To obtain a smaller fragment containing the pAL5000 origin of repli-

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cation, a fragment of 1,777-bp was amplified from pYUB12 (6) by PCR with the primers NheIpAL5000N2 and pAL5000CNheI. The amplified fragment was digested with *Nhe*I and inserted into the unique *Nhe*I site of pK18 or pK19. The resulting plasmids (version 0) were designated pNV18 and pNV19, respectively. Next, we eliminated the *Eco*RI and *Sal*I/*Hin*cII sites located in the pAL5000 origin of replication by site-directed mutagenesis, resulting in version 1 vectors (Fig. 1).

The transformation efficiency of *N. farcinica* with pNV vectors varied between experiments. When 100 ng of supercoiled pNV19 DNA propagated in *E. coli* was used, the efficiency ranged from 2.4×10^5 to 1.3×10^6/μg DNA. The stability of pNV vectors in a *Nocardia* strain was investigated. When pNV19-carrying *N. farcinica* IFM 10152 cells were grown in the absence of antibiotic, the number of neomycin-resistant cells decreased to about 10% of the population at the fourth subculture (Fig. 2). The slope of the regression line was −0.24, indicating that about a quarter of the population lost the plasmid at each subculture. However, no remarkable plasmid loss was observed in the presence of 50 μg/ml of neomycin (Fig. 2).

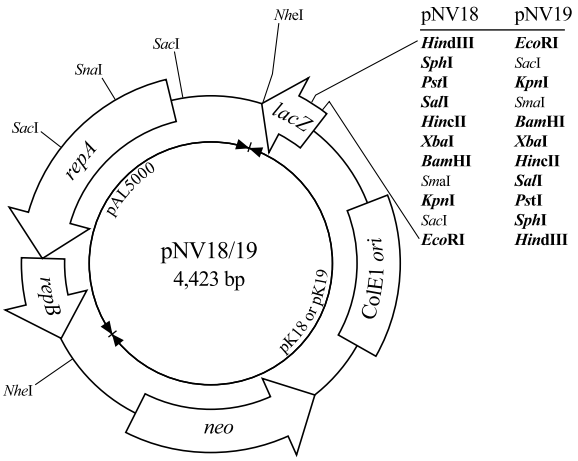

Fig. 1. Restriction maps of pNV18 and pNV19. The shuttle vectors pNV18 and pNV19 are composed of the origin of replication region from pAL5000 and the entire sequence of pK18 or pK19. Unique restriction sites in the multiple cloning site are indicated in bold type. The position and direction of genes are indicated by arrows. The complete nucleotide sequences of pNV18 and pNV19 have been deposited in the DDBJ database under accession numbers AB267085 and AB267086, respectively.

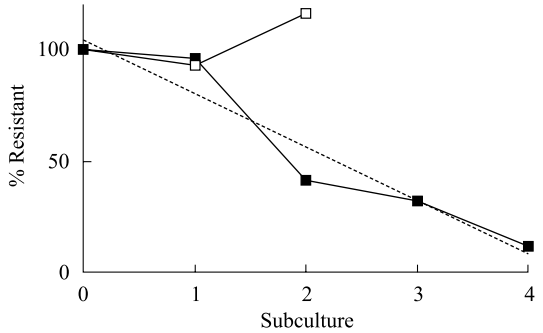

Fig. 2. Stability of pNV19 in *N. farcinica* IFM 10152. pNV19-carrying strain was grown in the absence (open symbol) or presence (closed symbol) of 50 μg/ml of neomycin. The plots show the percentage of neomycin-resistant cells in each subculture. The regression line for the subcultures without neomycin is indicated by a dotted line.

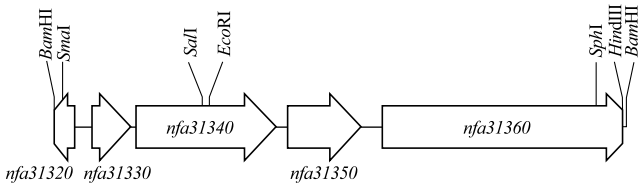

Fig. 3. Restriction map of the 3.8-kb *Bam*HI fragment containing the *nfa31340* gene. The position and direction of genes are indicated by arrows.

Table 1. Aminoglycoside resistance profiles of *Nocardia* strains

Strain/Plasmid	Resistance (μg/ml)	
	Tobramycin	Neomycin
N. farcinica IFM 10152	100	<5
N. asteroides IFM 0319^T	<5	<5
N. asteroides IFM 0319^T/pNV19	<5	50
N. asteroides IFM 0319^T/pNVaph(2")	50	50

Cloning of the tobramycin resistance gene of *N. farcinica*: To evaluate the new vectors, we attempted to clone the tobramycin resistance gene of *N. farcinica* IFM 10152. *Nocardia* spp. are intrinsically resistant to many antibiotics, and their drug resistance patterns are used for species identification (12). Resistance to tobramycin has been used to separate *N. farcinica* from *N. asteroides* complex (13). Previously, we reported that the *N. farcinica* IFM 10152 genome contains three putative aminoglycoside phosphotransferase genes (1). Among them, the *nfa31340* gene likely encodes an aminoglycoside *2*"-*O*-phosphotransferase (APH(2")), which can inactivate tobramycin. The deduced amino acid sequence of the *nfa31340* gene product shows marked homology with APH(2")-Ic (33% identity and 48% similarity) (14), APH(2")-Id (27% identity and 48% similarity) (15), APH(2")-Ie (26% identity and 47% similarity) (16), APH(2")-Ib (26% identity and 43% similarity) (17), and APH(2")-Ia (26% identity and 44% similarity) (18). These observations suggest that the *nfa31340* gene is responsible for the tobramycin resistance of *N. farcinica* IFM 10152.

The *nfa31340* gene is preceded by the *nfa31330* gene in the genome with an intergenic region of only 38 bp, suggesting that the *nfa31340* gene does not have its own promoter. Therefore, we cut a 3.8-kb *Bam*HI fragment containing both *nfa31330* and *nfa31340* genes (Fig. 3) out of the pKNL015_E03, a plasmid of the ordered plasmid library of the *N. farcinica* IFM 10152 genome, and cloned it into the *Bam*HI site of pNV19, yielding pNVaph(2"). Subsequently, pNVaph(2") was introduced into *N. asteroides* IFM 0319^T, which is susceptible to tobramycin. As shown in Table 1, *N. asteroides* IFM 0319^T carrying pNVaph(2") showed significant resistance to tobramycin in addition to neomycin, the resistance to which would be conferred by the vector alone. However, the resistance level of *N. asteroides* IFM 0319^T carrying pNVaph(2") was significantly lower (50 μg/ml) than that of *N. farcinica* IFM 10152 (100 μg/ml), suggesting the involvement of several mechanisms in resistance to tobramycin in *N. farcinica* IFM 10152.

DISCUSSION

The new shuttle vectors we produced in this study possessed the useful properties of pK plasmids. That is, *lacZ* with a multiple cloning site enables conventional blue/white

screening of recombinant clones in *E. coli*, and the Tn*5*-derived *neo* gene encoding an aminoglycoside *3*'-*O*-phosphotransferase (19) is expressed in both *Nocardia* and *E. coli*, and confers kanamycin and neomycin resistance on the host.

pAL5000 is one of the most widely used plasmids for the development of cloning vectors in *Mycobacterium*. Its origin of replication also works in *Nocardia* (3) and *Rhodococcus* (20). The stability of pAL5000-based plasmids in these two genera may be lower than in *Mycobacterium* (9). Mangan et al. reported that a pAL5000-based vector was also unstable in *Rhodococcus* when grown in the absence of antibiotics (20). However, pNV18 and pNV19 were stably maintained in the presence of antibiotics. We showed here that pNV vectors could carry a 3.8-kb DNA fragment. Moreover, pNV vectors have been shown to be able to clone a DNA fragment up to 7.3 kb in length in shotgun cloning experiments (unpublished), details of which will be described elsewhere. The host range of pNV vectors would be broad. As described above, pNV19 could replicate autonomously in *N. farcinica* and *N. asteroides*. In addition, *N. nova* and *N. cyriacigeorgica* could be transformed with pNV18 (J. Schloendorn and B. Rittmann, personal communication).

To our knowledge, pNV18 and pNV19 are the first cloning vectors derived for practical use in *Nocardia*. These vectors will facilitate molecular biological studies of *Nocardia* spp.

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