SHORT COMMUNICATION

Cloning and Characterization of the Replicon of the *Nocardia italica* Plasmid, pNI100

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A 19-kb plasmid, pNI100, was isolated from *Nocardia italica* CCRC12359; its replicon was cloned and characterized as having a single open reading frame (ORF) of 1188 bp specifying 396 amino acids (aa). Analyses of the deduced aa sequence of the Rep protein indicated that characteristics of three consensus sequences and a P-loop-like motif in the Rep protein of plasmid pSG5, a conjugative plasmid involving a rolling-circle replication mechanism, were conserved in those of plasmid pNI100. Phenotypically, a pock structure was produced in the regenerated mycelium by introducing pNI100 DNA into the *Streptomyces lividans* protoplast. This result strongly suggests that pNI100 is a conjugative plasmid and probably replicates by a rolling-circle replication mechanism. By using the replicon of pNI100, a bifunctional plasmid pNI105 that could replicate in both *Escherichia coli* and *S. lividans* was constructed and found to be a useful cloning shuttle vector. © 2000 Academic Press

Key Words: *Nocardia italica*; replicon; sequence; pock formation; shuttle vector.

Nocardia italica is a gram-positive, mycelial, soil bacterium belonging to the prokaryote family Actinomycetaceae. This microorganism has been used in a microbiological process to transfer a hydroxy group into the 16 α position of a steroid molecule (Amici *et al.*, 1966), a well-known intermediate in industry for preparing a variety of pharmaceuticals including antiphlogistic drugs (Wright *et al.*, 1960). The presence of plasmids in *Nocardia* species is well documented (Kirby and Usdin, 1985; Oh *et al.*, 1985). However, few of their replicons are characterized as a genetic cloning system for *Nocardia* species. Although Madon and Hutter (1991) developed a cloning system in which a recombinant plasmid, pMEA123, based on the indigenous plasmid pMEA100 of *Amycolatopsis* (*Nocardia*) *mediterranea*, was constructed and used as a cloning vector involved in a direct

transformation in the bacterial mycelia, its replicon was not isolated from the plasmid of authentic *Nocardia* species.

In the present study, the first reported isolation, characterization, and complete sequence of the replicon of pNI100 from *N. italica* CCRC 12359 (Culture Collection and Research, Hsinchu, Taiwan, R.O.C.) are given, and the deduced amino acid (aa)² sequence encoded by this replicon shows a high degree of similarity to that of *Streptomyces ghanaensis* pGS5 (Muth *et al.*, 1988, 1995). In another experiment, a recombinant plasmid, pNI105, based on the pNI100 replicon, was constructed and found to be useful as a shuttle cloning vector for the genetic study of *Nocardia* species.

Methods for DNA manipulation and techniques for isolating replicons of plasmid pNI100 from *N. italica* were used as described previously (Liu *et al.*, 1998) with modifications. Briefly, plasmid pNI100 was completely di-

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² Abbreviations used: aa, amino acid; ORF, open reading frame; Thio^r, thiostrepton resistant.

FIG. 1. Restriction map of pNI100 (19.4 kb) and the strategies used to clone the replicon of pNI100. The restriction sites and location of the replicon on pNI100 are indicated. The segments derived from pNI100 and pIJ702 are depicted as heavy lines and light lines, respectively, in pNI101. The restriction sites and locations of the replicon in both pNI101 and pNI105 are indicated.

FIG. 2. The nucleotide sequence of the replicon of *N. italica* plasmid pNI100. The deduced amino acid (single-letter code) is shown below the first nucleotide of each codon. The numbers at the top correspond to the nucleotides. A dyad symmetry sequence, which can form a stem-loop for the transcription termination site, is indicated. Horizontal arrows highlight the stem-loop.

gested with *Bcl*I, and the subsequent DNA fragments were ligated with the *Bcl*I DNA fragment bearing the thiostrepton resistance gene of plasmid pIJ702 (Katz *et al.*, 1983). The ligation mixture was used to transform the protoplasts of *S. lividans* 66, and the transformants were selected in the presence of thiostrepton according to the method described by Katz *et al.* (1983). To precisely locate the replication region in the *Bcl*I fragment of pNI100, the replicon was subcloned on pIJ702 by performing a series of deletion and insertion experiments. For construction of pNI105, the *Kpn*I digest of pNI101 (Fig. 1) was ligated with the linearized pBlue-script (SK⁺) cut at the *Kpn*I site. Transforma-

tion of *S. lividans*, *N. italica*, and *Escherichia coli* with the recombinant plasmid DNA was performed by the methods described by Hopwood *et al.* (1983) and Sambrook *et al.* (1989), respectively. Transformation frequencies referred to the number of transformant caused by 1 μ g of plasmid DNA. The nucleotide sequences of plasmid DNA were determined on both strands by primer walking, using the dideoxy chain termination procedure (Sanger *et al.*, 1977), and the homology search for the deduced amino acid sequence was done using DNAsis Software (Hitachi Software Engineering Co. Ltd., Japan). The nucleotide sequence of the pNI100 replicon has been deposited with

TABLE 1
Transformation Efficiency of the Recombinant Plasmids

Host cell	Plasmid	Transformation efficiency (CFU/ μ g)
<i>S. lividans</i> 66	pIJ702	2.0×10^5
<i>S. lividans</i> 66	pNI101	1.7×10^6
<i>S. lividans</i> 66	pNI105	2.3×10^5
<i>N. italica</i> CCRC 12359	pNI101	0
<i>N. italica</i> CCRC 12359	pNI105	0
<i>E. coli</i> JM109	pNI105	1.0×10^4
<i>E. coli</i> JM109	pBluescript (SK ⁺)	4.5×10^4

Note. Medium used for selecting transformants contained 50 μ g/ml of thiostrepton or ampicillin.

GenBank Nucleotide Sequence Data Library under Accession No. AF061277.

The experiment of pock formation was performed by transforming *S. lividans* TK24 protoplasts according to the method described by Hopwood *et al.* (1985). Initially, 5 μ l DNA solution (0.001 μ g pNI100 DNA) was added to 50 μ l of protoplast suspension (10^{10} /ml) and mixed by tapping the tube. Then, 200 μ l of 25% PEG 1000 in P buffer was added and mixed by pipetting. Subsequently, the protoplast suspension was spread on two R2YE plates. All plates were incubated at 28°C until the presence of pocks was noted.

The molecular size of plasmid pNI100 was calculated from fragment analysis and found to be 19.4 kb. Restriction endonuclease analyses revealed a single cleavage site for *Pst*I and multiple restriction sites for *Bcl*I (6), *Mam*I (3), *Kpn*I (3), and *Sma*I (4), mapped as shown in Fig. 1. Plasmid pNI100 has other cleavage sites for *Alf*III (2), *Nae*I (>3), *Pvu*II (3), *Bgl*I (>15), *Dra*II (>7), and *Apa*I (>10) (data not shown); however, it does not have cleavage sites for *Bam*HI, *Eco*RI, *Nhe*I, *Nsi*I, *Sca*I, *Ssp*I, *Sst*I, or *Xba*I. In the experiment for cloning the replication region of plasmid pNI100, a thiostrepton-resistant (Thio^r) transformant containing a plasmid pNI101 of about 5.7 kb was obtained. DNA analyses revealed that pNI101 consisted of a 4.7-kb *Bcl*I fragment of pNI100 and a 1.1-kb *Bcl*I fragment containing a thiostrepton resistance (*tsr*) gene of pIJ702 (Fig. 1). Since the *Bcl*I fragment containing the *tsr* gene of pIJ702 did not contain a replicon sequence, the result

that pNI101 could successfully replicate in *S. lividans* 66 indicated that: (1) pNI101 did acquire the replicon sequence of pNI100 and (2) the replicon of pNI100 could be recognized by the replicative machinery of *S. lividans* 66. Moreover, deletion and insertion experiments (data not shown) revealed a minimized fragment that spanned at least the 2.4-kb *Sma*I–*Sma*I fragment (Fig. 1) for carrying the replication functions of pNI100. Further evidence concerning the location and the size of the pNI100 replication region was obtained from DNA sequence analyses (Fig. 2).

In the construction of a pNI101-based bifunctional cloning vector, a chimeric plasmid pNI105 (Fig. 1) was created. This bifunctional plasmid pNI105 transformed *E. coli* to Ap^r and *S. lividans* 66 to Thio^r at a frequency as high as those of pBluescript (SK⁺) and IJ702, respectively; but it failed to transform *N. italica* (Table 1). We attributed this result to the possibility that the employed transformation conditions suitable for transforming *S. lividans* were not suitable for transforming *N. italica* or to the possibility that *N. italica* held strong DNA restriction activity to prevent the replication of foreign DNA (Madsen and Josephsen, 1998; Wilson and Murray, 1991). In addition, *N. italica* contained an endogenous plasmid, pNI100, that might be incompatible with the recombinant plasmid, pNI105, containing the same replicon as that of pNI100. In this investigation, we isolated the replicon of plasmid pNI100 from *N. italica* and constructed a shuttle vector, pNI105, with the same replicon. This

	10	20	30	40	50	60
Ni-pNI100	MWAAGEAALTGVCRCNNVHMCWCGSRILAVRAGNAQAMADGLAANGYGLHLGTSTLRHF					
	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::
Sg-pSG5	MMKPGEATWGNTCRCNNVHMCWCGSRILAVRGSNNVQLAADGLADAGYGLHLGTNTLRHF					
	10	20	30	40	50	60
	70	80	90	100	110	120
Ni-pNI100	ARMPFGSNRRQRFLVRLLDHGWGAYGSSGRPWRRKAEFGVIGYERAYEDTWGPDITG					
	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::
Sg-pSG5	ERMAFGTVRKGMRLHGLVAVLHDGWKAYGSSGRVRVTRMDDFGIIGYERAFEDTFGWGSG					
	70	80	90	100	110	120
	130	140	150	160	170	180
Ni-pNI100	HHLHWHTLWVTAEPDLDGQLEFRRAIATAWADGVPNAGGYEVSQTCDRPDCSCGGEHG					
	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::
Sg-pSG5	<u>WHLHWHTLWV</u> TREVLGPDQAQ-AFRDALAGAWAAGVESAGGYTVSETCDRPGCSCEGKGHG					
	<u>motif 2</u> 130	140	150	160	170	P-loop
	190	200	210	220	230	
Ni-pNI100	TDLRSL-----NSGEEGETARYLYKDG-----KGTAKIGLELSRQDLKDGRSGRLGPWQL					
	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::
Sg-pSG5	TDVRLNGADAADGDAGKQARYLYKDGDKTKGGVAKIGLELAGQNFKAGRGDDRMSPDL					
	180	190	200	210	220	230
	240	250	260	270	280	290
Ni-pNI100	GDAADELLASGGVPGPFITAYRERERERIGFVRKQYRTQGLNALVKSLEIPQHERTEDEI					
	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::
Sg-pSG5	GDAAAAE-LQRLRRPGPFVEKYRERERFVGQVRKHYSQNLNRLIKELGIQDQVREEEI					
	240	250	260	270	280	290
	300	310	320	330	340	
Ni-pNI100	TDESGGLQPIVIPSGTWYRTVARVPGR--VDLVKVAES-GVGRRLLIESWGLRWGID					
	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::	:: :: ::
Sg-pSG5	TDDTEGLVAIAVIPAYIWYRIARVAGRRLDLDLIKVAETYGLPGVRRLVESWGLVWGKD					
	300	310	320	330	340	350
	350	360	370			
Ni-pNI100	VFDPPAEPTTEPRRVRAEVD					
	:: :: ::	:: :: ::				
Sg-pSG5	VLDPPA-PEARPRRATLT/LR					
	360	370				

FIG. 3. Sequence homology between bacterial Rep proteins. Proteins are aligned to illustrate their homology. Conserved amino acids are indicated by a colon. Sequences are from the following sources: Ni-pNI100, *N. italica* plasmid pNI100; Sg-pSG5, *S. ghanaensis* plasmid pSG5 (Muth *et al.*, 1995). The numbers above and below the sequence indicate the number of amino acid residues in that protein. The consensus sequence motifs of initiation proteins for rolling-circle-type replication and the sequence resembling a P-loop-like nucleotide binding motif of pSG5 Rep protein (Muth *et al.*, 1995) are underlined and indicated.

useful shuttle DNA cloning vector contains two unique single cloning sites, *Bam*HI and *Eco*RI, and two selection markers, the ampicillin resistance gene for selecting the transformant of *E. coli* and the thiostrepton resistance gene for selecting the transformant of *S. lividans* (Fig. 1). This recombinant plasmid pNI105 provides the potential to further develop a cloning system that will facilitate the study of the molecular biology of *N. italica*.

An open reading frame (ORF) in the DNA fragment with replicative function from pNI100 was revealed by sequence analysis (Fig. 2). The coding region of the ORF has 1188 bp specifying 396 amino acids. A potential Rho-dependent transcription termination site (Rosenberg and Court, 1979), in which there is a region of dyad symmetry that can form a stem of 21 bp with a major loop of 9 nucleotides and a minor loop of 4 nucleotides (Fig. 2), is located downstream of the coding sequence. As shown in Fig. 3, the deduced amino acid sequence of the sin-

gle ORF of the pNI100 replicon exhibits 61.8% identity to the *rep* gene product of *S. ghanaensis* plasmid pSG5 (Muth *et al.*, 1995). This result indicates that the Rep protein of pNI100 is related to the Rep protein of plasmid pSG5. Furthermore, three consensus sequences (Fig. 3), LGTNTLRH (motif 1, aa 52–59), HLHWHTLWV (motif 2, aa 122–130), and QARYLYKDG (motif 3, aa 198–207), of the pSG5 Rep protein (Muth *et al.*, 1995) are highly homologous to those of the pNI100 Rep protein (Fig. 3). Since these motifs are characteristic of the initiation proteins mediating rolling-circle replication (Gruss and Ehrlich, 1989; Ilyina and Koonin, 1992), the consensus sequence suggests a rolling-circle replication mechanism (Gruss and Ehrlich, 1989; Novick, 1989) for plasmid pNI100. Moreover, another sequence with about 72% homology to the P-loop-like nucleotide binding motif of the pSG5 Rep protein, CDRPGCSCEGKGHGTD (aa 167–182), is also found in the Rep protein of plasmid

FIG. 4. Pocks produced by the growth of pNI100 transformed *S. lividans* TK24 in a lawn of plasmid-free *S. lividans* TK24. Pocks formed by the presence of a zone of lethal zygosis are indicated by arrows.

pNI100 (Fig. 3) although it is not as similar to the consensus P-loop motif described by Saraste *et al.* (1990). The P-loop nucleotide binding motif has been reported to be essential in the conjugation proteins of the *trwB* family (Saraste *et al.*, 1990; Cabezon *et al.*, 1994); therefore, it is not impossible that this sequence motif may be involved in the conjugative transfer of plasmid pNI100.

Many *Streptomyces* conjugative plasmids give rise to pocks formed by lethal zygosis reaction when a plasmid-carrying mycelium develops in a confluent lawn of mycelium of a strain lacking the same closely related plasmids (Hopwood *et al.*, 1985). Since *S. lividans* TK24 behaves as a largely nonrestricting strain and lacks endogenous plasmid, the plasmid DNA from other strains can be successfully transferred between the cells of *S. lividans* TK24. Thus, in this experiment, *S. lividans* TK24

CCRC 51704 was employed as an indicator strain for the pock reaction. As shown in Fig. 4, pocks were produced by the growth of pNI100-transformed *S. lividans* TK24 in the lawn of plasmid-free *S. lividans* TK24. This result suggests that pNI100 is a conjugative plasmid that can be successfully transferred between the cells of *S. lividans* TK24.

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