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Short Communication

Development of a vector and host system and characterization of replication of plasmid pSQ10 in moderately halophilic *Nocardiopsis*

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The genus of *Nocardiopsis* is a new source of antibiotics, chemicals, and enzymes. Here we reported the development of a vector and host system in moderately halophilic *Nocardiopsis* via an *oriT*-mediated conjugation. By screening about 80 *Nocardiopsis* strains, 6 of them harbored 8 plasmids (18–80 kb). The complete nucleotide sequence of pSQ10 consisted of 18,219 bp, with 71.9% G + C content, encoding 17 open reading frames, 5 of them resembled those of *Streptomyces* plasmids. A *rep* locus (iteron within the gene) was identified for replication in *Nocardiopsis* sp. YIM 90083, and rep protein bound to its iteron sequence. This system may be useful for gene cloning and expression in *Nocardiopsis*.

Keywords *Nocardiopsis*; plasmid; replication

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Introduction

Meyer [1] first described a new genus *Nocardiopsis* as a non-streptomycete group of actinomycete. Its mode of sporulation, DNA G + C content (64%–73%) and chemotaxonomic characteristics (e.g. cell-wall chemotype III/C, phospholipid type PIII, menaquinone MK-10, and fatty acid type 3d) are characterized [2]. About 30 species and subspecies have validly published names [3]. Most of them are isolated from saline or alkaline habitats [4], while some species were also found in antarctic glacier, marine sediment, actinorhizal plant rhizosphere, and gut tract of animals [5–7]. Recently, Sun *et al.* [8] published the complete genome sequence of *Nocardiopsis* dassonvillei type strain (IMRU 509^T). The 6,543,312 bp genome consists of a 5.77 Mbp circular chromosome with 73% G + C and a 0.78 Mbp plasmid with 72% G + C content.

A number of new antibiotics, chemicals, and enzymes have been identified in *Nocardiopsis* species. Griseusin D, a pyranonaphthoquinone antibiotic, showed strong cytotoxicity against human leukemia cells [9]. Nocardiopsins A and B, new macrolide polyketides, exhibited low-micromolar binding to immunophilin FKBP12 [10]. Apoptolidins E and F are two new glycosylated-macrolactones [11]. Four 3-methyl-4-ethylideneproline-containing peptides, lucentamycins A–D, showed significant *in vitro* cytotoxicity against human colon carcinoma [12]. Fijiolides A and B displayed activities on inhibition of TNF-α-induced NF-κB [13]. The marine *Nocardiopsis lucentensis* MSA04 produces a glycolipid biosurfactant, potential for bioremediation processes [14]. *Nocardiopsis aegyptia* degraded poly(3-hydroxybutyrate) and its copolymers [15]. *Nocardiopsis* sp. F96 produced a novel β-1,3-glucanase [16]. However, lack of a gene cloning and expression system in *Nocardiopsis* has impeded further investigation of these novel genes/gene clusters.

Here, we report the development of a vector and host system in *Nocardiopsis*, and the characterization of replication and inheritance of plasmid pSQ10.

Materials and Methods

Bacterial strains, plasmids, and general methods

About 80 moderately halophilic *Nocardiopsis* strains designated YIM 90002, etc., isolated from varied saline soils of Xinjiang Uygur Autonomous Region of China, and identified by the standard procedures of actinomycete classification, were provided by Chenglin Jiang and Lihua Xu (Yunnan University, Kunming, China). *Nocardiopsis* strains were cultured on ISP5 medium supplemented with 10% NaCl [*L*-asparagine, 1 g; glycerol, 10 g; K_2HPO_4, 1 g; NaCl, 100 g; 1 ml of the trace element solution ($FeSO_4.7H_2O$, 1 g; $MnCl_2.4H_2O$, 1 g; $ZnSO_4.7H_2O$, 1 g;

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1 l of H_2O,); agar, 20 g; and 1 l of H_2O, pH 7.2]. Pulsed-field gel electrophoresis, preparation of protoplasts and transformation of *Streptomyces lividans* ZX7 [17] followed Kieser *et al*. [18]. Electroporation of *Rhodococcus* sp. N1037 followed Shen *et al*. [19]. *Escherichia coli* strains DH5α [*supE*44, Δ*lac*U169 (ϕ80*lacZ*ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1; Life Technologies, Carlsbad, USA] and ET12567 (*dam dcm hsdM cm kan*) was used as cloning hosts. Plasmids pSP72 (Life Technologies) and pHY1 were used as cloning vectors. Plasmid isolation and transformation followed Sambrook *et al.* [20].

Isolation of *Nocardiopsis* circular plasmids

Isolation of *Nocardiopsis* circular plasmids followed a protocol of preparation of *Streptomyces* plasmids [21] with slight modification. About 50 mg mycelium was suspended in 500 μl lysozyme solution (2 mg/ml lysozyme, 10.3% sucrose, 25 mM Tris-HCl, 25 mM EDTA, pH 8) at 37°C for 30 min. About 250 μl of 0.3 M NaOH/2% SDS solution was added, mixed thoroughly and incubated at 55°C for 30 min. After cooling, the DNA solution was extracted twice with 250 μl neutral phenol/chloroform (in 0.1 M Tris-HCl, pH 8), and was centrifuged at 12,000 *g* for 10 min. DNA was precipitated from the supernatant with isopropanol, washed twice with 70% ethanol, and dissolved in 15 μl TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) buffer.

DNA sequencing and analysis

The 16S rRNA genes of the *Nocardiopsis* strains were amplified by polymerase chain reaction (PCR) with primers (5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-TCAGGC TACCTTGTTACGACTT-3′). PCR conditions were: template DNA denatured at 95°C for 5 min, then 95°C 30 s, 55°C 30 s, and 72°C 2 min, for 35 cycles. A phylogenetic tree was generated by using a neighbor-joining method [22].

Shotgun cloning and sequencing of pSQ10 were performed on the Genome Sequencer FLX 454 System (Roche, Basel, Switzerland) at the Chinese Human Genome Center in Shanghai (Shanghai, China). Analysis of protein coding regions was performed with 'FramePlot 3.0 beta' (http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl) [23]. Sequence comparisons and protein domain searching were done with software from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). DNA secondary structure was predicted with 'DNA folder' (www.bioinfo.rpi.edu/applications/mfold/old/dna/) and 'Clone Manager version 5' (http://clone-manager.software.informer.com/). The GenBank accession number for the complete nucleotide sequences of pSQ10 is DQ399904.

Transformation of *Nocardiopsis* via conjugation

pSQ10-derivied plasmids were introduced by transformation into *E. coli* ET12567 containing pUZ8002 [18] and cultured on Luria–Bertani (LB) medium (supplied with 50 μg/ml kanamycin, 50 μg/ml chloramphenicol, and 100 μg/ml ampicillin) at 37°C for 16 h. The culture (1 ml) was transferred into 50 ml fresh LB medium containing the antibiotics and incubated for another 1–2 h until the absorption at 600 nm reached 0.6. Then, cells were harvested and washed with cold LB medium. *Nocardiopsis* spores were suspended in YT (yeast extract, 10 g; tryptone, 16 g; NaCl, 5 g; and 1 l of H_2O, pH 7) liquid medium and incubated at 50°C for 10 min and then at 30°C for 2 h. About 10^{10} *E. coli* cells were mixed with 10^{10} *Nocardiopsis* spores on MS medium (mannitol, 20 g; soya flour, 20 g; agar, 20 g; and 1 l of H_2O) supplemented with 1% NaCl and 25 μg/ml of nalidixic acid and cultured at 30°C for 24 h, and then transconjugants were selected by incubation in 50 μg/ml of thiostrepton for 3–5 days.

Electrophoretic mobility shift assay

The pSQ10 *repA* gene (9867–10,944 bp) was cloned in an *E. coli* plasmid pET28b (Novagen, Gibbstown, USA) to obtain pAZ128 and then introduced into *E. coli* strain BL21 (DE3) (Novagen). Then, 0.5 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) was added into a log-phase LB culture at 16°C and cultured for 12 h to induce overexpression of the cloned gene. The His_6-tag repA protein was purified to ~90% homogeneity by Ni^{2+} column chromatography according to the supplier's instructions (Qiagen, Hilden, Germany). The 240-bp DNA sequence (9961–10,200 bp) containing the pSQ10 iteron was amplified by PCR and inserted into the *Xho*I site of pSP72 to construct pAZ143. The 240-bp DNA was released by treatment of pAZ143 with *Xho*I and was end-labeled with [α-^{32}P]dCTP using DNA polymerase Klenow fragment. The DNA-binding reaction was performed at room temperature for 10 min in the buffer (10 mM Tris-HCl, pH 7.5, 25 mM KCl, and 10% glycerol). The reaction complexes were separated on a 5% pre-run native acrylamide gel in 0.5 × Tris-borate-EDTA buffer at 150 V for 2 h. The gel was dried and analyzed using the Phosphorimager (Fuji, Tokyo, Japan).

Results

Detection of plasmids among moderately halophilic *Nocardiopsis* strains

Eighty moderately halophilic actinomycete strains (growing on ISP5 media supplemented with 1%–10% NaCl and producing spores at 30°C) were isolated. By using a slightly modified protocol for isolation of *Streptomyces* plasmid, eight circular plasmids of six strains (YIM 90127, 90115, 90136, 90147, 90201, and 90035), ranged in sizes from 18–80 kb (**Table 1**) were detected on 0.7% agarose gels. By using the pulsed-field gel electrophoresis, no linear

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conformation of DNA was observed, suggesting absence of linear plasmids among these strains.

To determine whether these plasmid-harboring strains were classified in a genus of *Nocardiopsis*, 16S rRNA genes were PCR-amplified and sequenced. The sequences displayed high similarity (99%) to these of known *Nocardiopsis* species. A phylogenetic tree was drawn by using a neighbor-joining method. As shown in **Fig. 1**, two strains (YIM 90115 and 90136) resembled *Nocardiopsis dassonvillei* sub sp. dassonvillei D21, and four strains (YIM 90127, 90147, 90035, and 90201) resembled *Nocardiopsis terrae* YIM 90022, *Nocardiopsis exhalans* XMU29, and *Nocardiopsis quinghaiensis* YIM 28A4.

Table 1 Detection of circular plasmids among 80 *Nocardiopsis* strains

Strain number	Sizes (kb) and names of the plasmids detected
YIM 90127	18 (pSQ10), 70 (pSQ9)
YIM 90115	25 (pSQ11)
YIM 90136	30 (pSQ13), 35 (pSQ12)
YIM 90147	50 (pSQ14)
YIM 90201	80 (pSQ15)
YIM 90035	60 (pSQ16)

The complete nucleotide sequence and analysis of plasmid pSQ10

About 18-kb pSQ10 of strain YIM 90127 was the smallest one among the detected plasmids. pSQ10 was treated with restriction endonucleases including *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I, and *Sac*I, and unique sites of *Bgl*II and *Hin*dIII in plasmid were determined. The *Hin*dIII-digested DNA was cloned in an *E. coli* plasmid pSP72 and completely sequenced. The insert sequence in pSP72 consisted of 18,219 bp, with 71.9% G + C content, resembling that of typical *Nocardiopsis* genes (e.g. 73% for the *Nocardiopsis dassonvillei* IMRU 509^T genome). Seventeen open reading frames (ORFs; pSQ10.1c–pSQ10.17c) were predicted by 'FramePlot 3.0 beta'. Among them, 5 of them resembled genes of known functions, 1 was hypothetical and 11 were unknown genes (**Table 2**). Interestingly, pSQ10.10 resembled rep protein (identity 78/205; 38%) of *Streptomyces* plasmid pCQ3(NC_013449; [24]), and pSQ10.12c resembled ParA protein of *Streptomyces* plasmid pRL2 (expectation value is 4×10^{-19}, identity 70/217; 32%), suggesting these *loci* for plasmid replication and inheritance. pSQ10.2c, containing a domain of DNA segregation ATPase FtsK/SpoIIIE, resembled a major conjugation protein TraA (identity 168/557; 30%) of *Streptomyces* plasmid pZL12 (NC_013420, [24]). Additionally, pSQ10.13, containing a domain of DNA

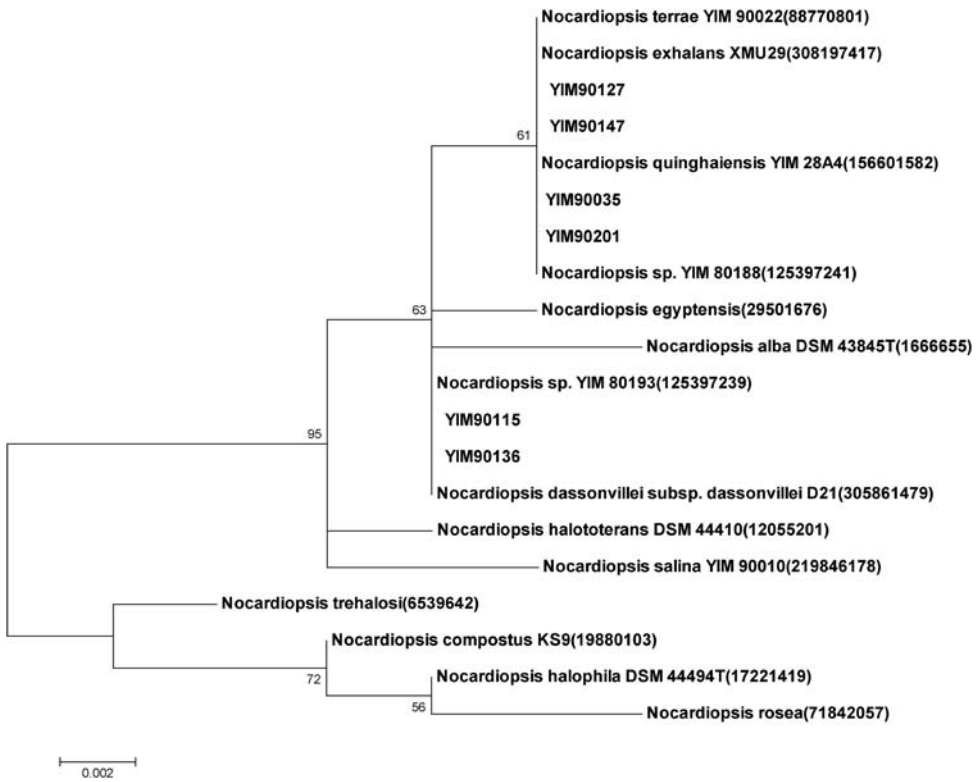

Figure 1 Phylogenetic tree for 6 newly identified strains and 14 known *Nocardiopsis* species (GenBank numbers in parentheses) The tree is drawn to scale using a neighbor-joining method, with branch lengths in the same units as those of the evolutionary distances. Numbers next to the branches are the percentage of replicate trees (the bootstrap test is 500 replicates).

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Table 2 Predicted opening reading frames in plasmid pSQ10

ORF	Position (bp)	Size (aa)	Expectation value	Similarity/functions (organisms)
pSQ10.1c	508–98	136		Unknown
pSQ10.2c	2745–505	746	5×10^{-32}	TraA (*Streptomyces* plasmid pZL12)
pSQ10.3c	4470–2779	563		Unknown
pSQ10.4c	4671–4483	62		Unknown
pSQ10.5c	5153–4668	161	4×10^{-36}	Hypothetical protein (*Nocardiopsis*)
pSQ10.6c	6844–5189	551		Unknown
pSQ10.7c	7582–6944	212		Unknown
pSQ10.8c	8155–7853	100		Unknown
pSQ10.9	8493–9269	258	6×10^{-39}	KorA (*Streptomyces*)
pSQ10.10	9499–10,944	481	2×10^{-24}	Rep (*Streptomyces* plasmid pCQ3)
pSQ10.11c	11,477–11,130	115		Unknown
pSQ10.12c	12,100–11,477	207	4×10^{-19}	ParA (*Streptomyces* plasmid pRL2)
pSQ10.13	12,474–14,558	694	1×10^{-47}	Relaxase (*Streptomyces* plasmid pFP1)
pSQ10.14c	15,672–14,851	273		Unknown
pSQ10.15c	16,284–15,688	198		Unknown
pSQ10.16c	16,735–16,493	80		Unknown
pSQ10.17c	17,953–17,405	182		Unknown

c, complementary strand.

nickase or relaxase, resembled TraI of bacterial plasmid transfer genes (e.g. *Streptomyces*, *Mycobacterium*, and *Stackebrandtia*). These results suggested that the replication, inheritance, and transfer genes of *Nocardiopsis* plasmid pSQ10 resembled those of *Streptomyces* plasmids.

Development of a vector-host system via an *oriT*-mediated conjugation in *Nocardiopsis*

To develop a gene cloning system in *Nocardiopsis*, 12 well-sporulating strains were selected. Nine (YIM 90021, 90037, 90075, 90083, 90132, 90149, 90151, 90207, and 90233) of them could not grow on ISP5 medium supplied with 50 μg/ml of thiostrepton, while three (YIM 90107, 90114, and 90156) were resistant to this antibiotic. Full length of pSQ10 was cloned in an *E. coli* vector pHY1 [**Fig. 2(A)**] containing a *tsr* (thiostrepton resistant) gene and an initiation site *oriT* of plasmid transfer to obtain pSQ23, and was introduced by conjugation from *E. coli* ET12567 containing pUZ8002 to *Nocardiopsis* strains. Thiostrepton-resistant colonies were obtained for strains 90083 and 90149, and no transformant for seven other strains.

pSQ23 was also introduced by PEG-mediated transformation of protoplasts or electroporation of mycelia of strain 90083. However, no transformants were observed.

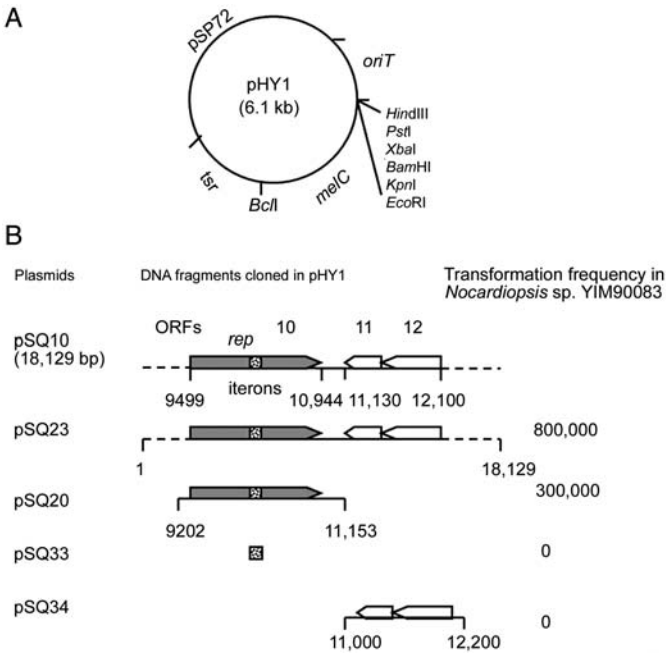

Figure 2 Identification of a pSQ10 locus for replication in *Nocardiopsis* (A) Schematic map of plasmid pHY1. Functional region/genes and relevant restriction enzyme sites are indicated. (B) Identification of a pSQ10 locus for replication in *Nocardiopsis*. Plasmids were constructed in *E. coli*, and introduced by conjugation from *E. coli* ED12567 into *Nocardiopsis* sp. YIM 90083. Positions of these cloned fragments on pSQ10 and transformation frequencies (transformants/μg DNA) are shown. Iterons are indicated by dotted boxes, replication gene by filled arrowheads and other relevant genes by open arrowheads.

Characterization of replication and inheritance of pSQ10

Since pSQ10.10 resembled rep of *Streptomyces* plasmid pCQ3 (**Table 2**), its gene was cloned in pHY1 to obtain pSQ20 and introduced by conjugation into strain 90083. As shown in **Fig. 2(B)**, like pSQ23, pSQ20 could also

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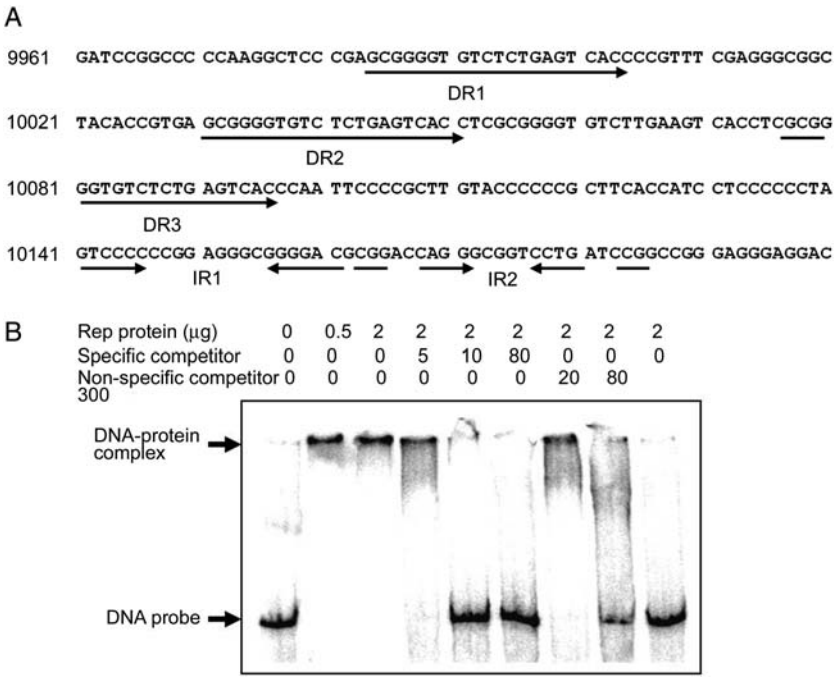

Figure 3 Examination of the binding activity of the pSQ10 Rep protein with its iteron DNA (A) Iteron of pSQ10. Possible iteron sequences on pSQ10 are shown. Direct repeat (DR) and inverted repeat (IR) sequences are indicated by arrowheads. (B) Examination of the binding activity of the pSQ10 Rep protein with its iteron DNA by the EMSA. The DNA probe for each lane was 5 ng, the probe also as specific competitor and salmon sperm DNA as non-specific competitor, and the numbers equal the fold of 5 ng. DNA-protein complexes are indicated.

efficient transform strain 90083, while no transconjugants were obtained for pHY1 containing an iteron within pSQ10.10 (pSQ33) or its adjacent two genes (pSQ34). To investigate whether pSQ23 could also propagate in *Streptomyces* and other actinomycete species, transformation of protoplasts of *S. lividans* ZX7 and electroporation of *Rhodococcus* sp. N1037 were performed, but no transformants were obtained. Thus, pSQ10.10 (designed *rep*) was a replication gene in *Nocardiopsis*.

Inheritance of pSQ20 and pSQ23 were measured. After one round of growth (5 days) on MS medium (supplied with 2% NaCl) without antibiotic selection, inheritance of two plasmids in strain 90083 were 0.1 and 90%, respectively. Similar frequencies were obtained for the plasmids in strain 90149 (0.1% and 87%, respectively). These results suggested that a *parA* locus (pSQ10.11c–pSQ10.12c) might involve in inheritance of pSQ10.

A ~200-bp iteron sequence (three direct repeats and two inverted repeats) was predicted within *rep* of pSQ10 by 'DNA Folder' and 'Clone Manager version 5' [**Fig. 3(A)**]. To investigate the possible interaction between the rep protein and iteron sequence, electrophoretic mobility shift assay (EMSA) for DNA-protein complex formation was performed. As shown in **Fig. 3(B)**, the 'super-shift' DNA bands were visualized with adding rep protein, indicated that the rep protein could bind to the DNA probe and formed a large DNA-protein complex. Formation of this complex was inhibited completely by adding a 10- and 80-fold excess of unlabeled probe or a 300-fold excess of salmon sperm DNA as non-specific competitor, indicating that the specificity of the rep protein binding to the iteron DNA was low.

Discussion

A few genes have been isolated from *Nocardiopsis* species, and have to be confirmed in other bacterial hosts such as *Streptomyces* and *E. coli*. For examples, a chitinase B gene from *N. prasina* OPC-131 is expressed in *S. fradiae* SU-1 [25]; a β-1,3-glucanase gene from alkaliphilic *Nocardiopsis* sp. F96 is cloned and is functionally expressed in *E. coli* [16]. A thiopeptide antibiotic, TP-1161, biosynthetic gene cluster from marine *Nocardiopsis* was confirmed through the targeted gene inactivation in the original host, but attempts at heterologous expression of the gene cluster in *S. coelicolor* failed [26]. A gene cluster for biosynthesis of K-252a (an unusual dihydrostreptose moiety cross-bridged to K-252c aglycone with two C–N linkages) from *Nocardiopsis* sp. K-252 (NRRL15532) is found, and has to be demonstrated by *in vitro* heterologous expression system of *E. coli* [27]. Here, we developed a vector and host system, which might help gene cloning and expression in *Nocardiopsis*, especially those useful antibiotic biosynthetic gene clusters and genes encoding alkaliphilic proteins.

Of nine *Nocardiopsis* strains, two (YIM 90083 and 90149) can be transformed with pSQ23, indicating a

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narrow host range of this plasmid. More *Nocardiopsis* plasmids would be investigated to find a plasmid with a wide host range. Strain YIM 90083 can be transformed at a high frequency via an *oriT*-mediated conjugation, since single-stranded DNA act as an intermediate during this process, we still do not know if this strain could restrict entrance of double-stranded DNA, in which the processes of electroporation- or PEG-mediated transformation of protoplasts involve. The new method following in *Streptomyces* to establish a gene disruption and replacement system in *Nocardiopsis* is worth to be investigated in future [28]. Although the *rep* locus of *Nocardiopsis* plasmid pSQ10 resembles that of *Streptomyces* plasmid pCQ3, pSQ10-derived plasmid (e.g. pSQ23) cannot propagate in *S. lividans*, suggesting difference in regulation of gene expression between the two species (e.g. Sigma factors of *Streptomyces* RNA polymerase might not recognize the transcription sites of the *Nocardiopsis* genes).

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