

Characterization of a Small Cryptic Plasmid from *Pseudomonas nitroreducens* Strain TX1

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Pseudomonas nitroreducens TX1에 존재하는 작은 플라스미드의 특성 규명

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Pseudomonas nitroreducens TX1 was isolated from a rice field drainage in Taiwan. The bacterium is of special interest because of its capability to use a group of nonionic surfactants such as alkylphenol polyethoxylates even at high concentrations as a sole carbon source. In this study, a small cryptic circular plasmid, pTX1, was characterized from *P. nitroreducens* TX1. It is 2,286 bp in length with a GC content of 63.3% and harbors three open reading frames, Rep_{pTX1} and functionally unidentified ORF1 and ORF2. The predicted rep_{pTX1} gene product is homologous to Rep proteins of plasmids belonging to the pC194/pUB110 family, which is predominantly found in Gram-positive bacteria and is known to replicate by the rolling-circle mechanism. The copy number of pTX1 was estimated to be about 150 in each cell. Based on the genetic fingerprints and comparison with other plasmids, it is concluded that pTX1 replicates by a rolling circle mechanism which is rarely found for *Pseudomonas* plasmids.

Keywords: *Pseudomonas nitroreducens*, cryptic plasmid, rolling circle replication

Circular plasmids are known to replicate by three mechanisms: theta type (bidirectional replication), strand displacement and rolling circle (RC) (del Solar *et al.*, 1998). RC replication plasmids present at a multicopy state in cells are generally small and the components necessary for replication of plasmid are tightly organized. Three critical components contained in all RC plasmids are the replication (Rep) protein, double strand origin (DSO), and single strand origin (SSO) (Khan, 1997). RC replication generally involves two main stages, leading strand replication and lagging strand replication (Khan, 1997). Rep protein is responsible for the initiation of replication and binds to DSO of the plus strand located in a

region with direct repeats and/or inverted repeats (Wang *et al.*, 1993). Based on sequence similarities in the Rep protein and DSO, RC plasmids can be grouped into six families including pT181, pR194/pLS1, pC194/pUB110, pSN2, pIJ101/pJVI, and other RC plasmids (Khan, 1997). Recently, a wider distribution of RC plasmids has been reported. Most known RC plasmids belong to Gram-positive bacteria, but are sporadically described for Gram-negative bacteria (Khan, 1997; Holtwick *et al.*, 2001). Especially in *Pseudomonas*, only *P. putida* P8 and *Pseudomonas* sp. S-47 strains are known to contain RC plasmids (Holtwick *et al.*, 2001; Chae *et al.*, 2005).

The genus *Pseudomonas*, Gram-negative bacteria, is widely distributed in the environment and plays an important role in biodegradation and biotransformation of organic and xenobiotic compounds (Gibson *et al.*, 1970; Yen and Serdar, 1988). *Pseudomonas nitroreducens* TX1 was isolated from a rice field

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drainage in Taiwan. The bacterium is of special interest because of its capability to use alkylphenol polyethoxylates, potential endocrine disrupters, as a sole carbon source even at high concentrations (Chen *et al.*, 2004, 2005, 2006; Lin *et al.*, 2010; Tuan *et al.*, 2011; Huang *et al.*, 2014). In this study, we report on the occurrence of a plasmid in strain TX1, named pTX1. On the basis of sequence analysis and comparison with known RC plasmids, we demonstrate that pTX1 replicates via a RC replication mechanism.

Materials and Methods

Bacterial strains, growth conditions, and recombinant DNA techniques

Strains TX1 and *Escherichia coli* DH5 α was routinely grown at 30°C and 37°C, respectively, in Luria-Bertani (LB) with antibiotic when required as previously described (Tuan *et al.*, 2011). Plasmid from *P. nitroreducens* TX1 was purified with a plasmid purification kit (NucleoGen Co., Korea). The plasmid, pTX1, was cut by *Sma*I and the resulting three restriction fragments isolated from 1% agarose gel electrophoresis were cloned into pUC19 (NEB Biolabs). The complete nucleotide sequence of plasmid was determined by primer walking from the resulting hybrid plasmids. The circular nature of pTX1 was also confirmed by PCR. The nucleotide sequence was determined by SolGent Co. Ltd (Korea) using an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.).

In silico analysis

ORFs were identified using a GETORF program provided by the Pasteur Institute (<http://bioweb.pasteur.fr/seqanal/interfaces/getorf.html>). The nucleotide and deduced amino acid sequences were compared with GenBank database using the basic BLAST Algorithm (<http://www.ncbi.nlm.nih.gov>). Multiple sequence

alignments were performed using Clustal X, version 2.0.3 using its default values (Thompson *et al.*, 1997). Repetitions within the nucleotide sequence were detected using a palindrome program (<http://emboss.bioinformatics.nl/cgi-bin/emboss/palindrome>). Promoter prediction program were used to identify promoter regions (http://www.fruitfly.org/seq_tools/promoter.html).

Determination of the plasmid copy number in *P. nitroreducens* TX1

Total chromosomal and plasmid DNA were extracted from TX1 strain using the procedure by Wilson (2003). Briefly, the cells were suspended in TE buffer (10mM Tris-HCl; pH 8.0 and 1 mM EDTA; pH 8.0) containing a final concentration of 100 μ g/ml proteinase K and 0.5% sodium dodecyl sulfate. DNA was recovered by isopropanol precipitation and re-dissolved in TE buffer containing 10 μ g/ml RNase. Total DNA was then diluted 1:10 and 1:100 with TE buffer, and subjected to 0.8% agarose gel electrophoresis at 100 V for 2 h. Comparison of the band densities between chromosomal and plasmid DNA was made using Quantity One software (Version 4.4.1, Bio-Rad). Plasmid copy number per cell was estimated using the method described by Quin *et al.* with a calculation of [chromosomal DNA size (6,700,249 bp)/ plasmid DNA size] \times (intensity ratio of plasmid versus chromosomal DNA bands) (Qin *et al.*, 2003; Huang *et al.*, 2014).

Nucleotide sequence accession number

The nucleotide sequence of the pTX1 has been deposited in GenBank with an accession number KJ948112.

Results

General characterization of the cryptic pTX1 plasmid

Plasmid pTX1 was initially detected on agarose gel during isolation of total genomic DNA from strain TX1. The copy

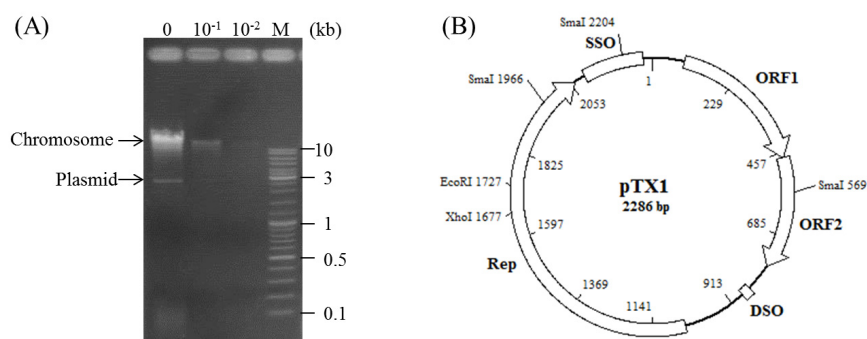


Fig. 1. Characterization of pTX1 in *P. nitroreducens* TX1. (A) Determination of pTX1 plasmid copy number. Total DNA was isolated and diluted 10–100 times and electrophoresed; M, size marker. (B) Genetic map of plasmid pTX1. Transcriptional directions of genes are indicated by arrowheads and relevant enzymes sites are shown. Rep, replication protein; SSO, single strand origin; DSO, double strand origin; ORF, open reading frame.

number of pTX1 plasmid in *P. nitroreducens* TX1 was estimated to be about 150 copies per cell (Fig. 1A). The plasmid pTX1 is 2,286 bp in length with a GC content of 63.3%. A genetic map with the elements harboring three putative ORFs is presented in Fig. 1B, whilst the corresponding sequence, emphasizing features of interest, is given in Fig. 2. Analyses of ORFs longer than 100 amino acid codons preceded by a putative ribosome binding site (RBS) revealed three genes

on the same strand in pTX1, namely *rep_{pTX1}*, *orf1*, and *orf2* with recognized promoters upstream of *rep_{pTX1}* and *orf1* (Fig. 2). The *rep_{pTX1}* gene of pTX1 encodes a protein of 337 amino acids that shares the highest amino acid sequence identity (84%) to putative replication protein of pPP8-1 (GenBank accession no. NP_064737) from *P. putida* P8 which is known to possess three cryptic circular plasmids (Holtwick *et al.*, 2001). A comparison of Rep protein from pTX1 to Rep proteins encoded

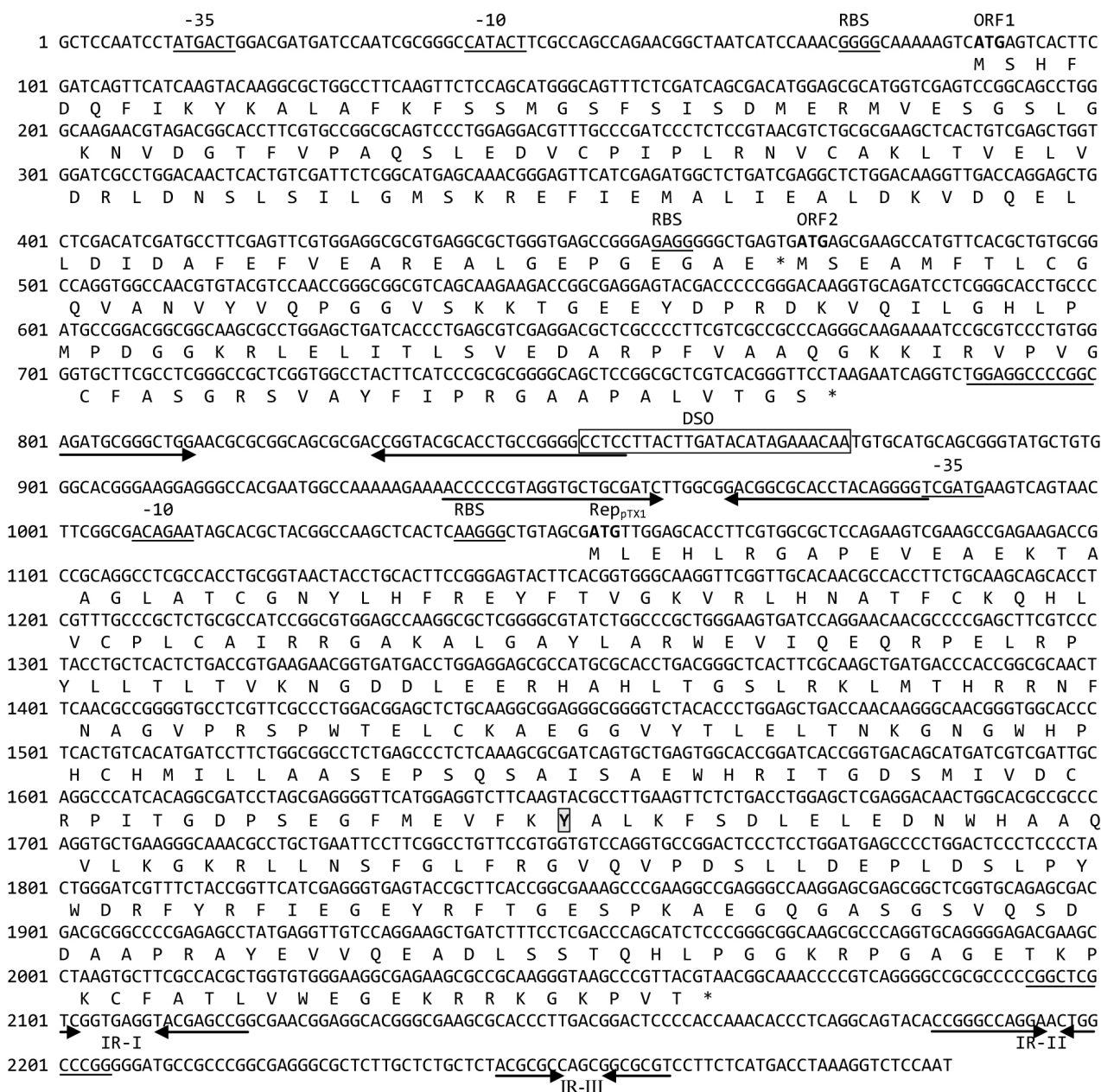


Fig. 2. Complete nucleotide sequence of pTX1. Potential ribosome binding site (RBS) sequences are underlined. ATG start codons are indicated in bold letters. Amino acids deduced from the nucleotide sequence are specified using the standard one-letter abbreviations. The boxed sequence corresponds to DSO, whereas inverted repeats characterizing the putative SSO are numbered and marked below the corresponding sequence (IR-I~III). Predicted active site of Rep_{pTX1} is indicated in gray-boxed Y.

<i>Pseudomonas nitroreducens</i>	pTX1	194-	M	E	V	F	K	Y	A	L	K	F	S	D	L	-208	
<i>Pseudomonas putida</i>	pPP8-1	195-	M	E	V	F	K	Y	A	V	K	F	S	D	L	-209	
<i>Staphylococcus aureus</i>	pC194	211-	Y	E		M	A	K	Y	S	G	K	D	S	D	Y	-225
<i>Staphylococcus aureus</i>	pUB110	242-	D	E	T	A	K	Y	P	V	K	D	T	D	F		-256
<i>Bacillus subtilis</i>	pBAA1	213-	L	E	I	S	K	Y	P	V	K	D	T	D	V		-227
<i>Lactobacillus plantarum</i>	pLP1	223-	E	E	T	A	K	Y	E	V	K	S	A	D	Y		-237
<i>Clostridium butyricum</i>	pCB101	261-	A	E	L	F	K	Y	M	T	K	V	T	G	E		-275
<i>Shigella sonnei</i>	pKYM	231-	A	E	T	L	K	Y*	S	V	K	P	E	D	M		-245
<i>E. coli</i> ssDNA Phage	φX174	341-	F	Y	V	A	K	Y*	V	N	K	K	S	D	M		-355

Fig. 3. Conservation of the amino acid sequence around Tyr-200 in Rep proteins from pTX1, the pC194/pUB110 family and the gene A protein of coliphages ϕ X174. Amino acids common to Rep_{pTX1} are boxed and highly conserved region is highlighted in grey. The tyrosine residues marked with asterisks are described as the linkage sites to the DNA when nicking occurs at the origin. Amino acid positions of sequences are given according to the published Rep sequences.

by other plasmids shows that it has homology to Rep proteins of the pC194/pUB110 plasmid family which is predominantly found in Gram-positive bacteria and *E. coli* ssDNA phages (Khan, 1997). The predicted proteins ORF1 and ORF2 with 127 and 101 amino acids, respectively, have no significant similarity with known proteins in database and share 45% and 77% similarity to ORF B and C of pPP8-1, respectively. Downstream of the *orf1* and *rep_{pTX1}* genes, inverted repeats were found from position 788 to 868 and 2,094 to 2,268, respectively. No potential terminator and promoter sequences could be identified downstream of *orf1* and upstream of *orf2*, respectively, indicating co-transcription of the two genes.

Conservation of the amino acid sequence around Tyr-200 in Rep_{pTX1} protein

The importance of Lys-Tyr region of Rep proteins in plasmid multiplication has been demonstrated in several studies, in which tyrosine residue acts as an acceptor for the 5' end of the single-strand break introduced by the Rep protein (Bouia *et al.*, 1989; Yasukawa *et al.*, 1991). A comparison of amino acid sequence around Lys-Tyr region (Tyr-200, tyrosine at position 200) of Rep protein from pTX1 to Rep proteins

encoded by the pC194/pUB110 plasmid family revealed significant homology as shown in Fig. 3. Interestingly, the conserved amino acid sequences around Lys-Tyr of pTX1 and pPP8-1 are almost identical. The result suggests that Tyr-200 of Rep_{pTX1} might also play an important role in its function.

Identification of the DSO of replication

The DSO of replication is analyzed to be located immediately upstream of the *rep* genes at position 850-877 in pTX1 (Fig. 2). As depicted in Fig. 4, the DSO of pTX1 showed homology with the DSOs of the pC194/pUB110 plasmid family and contains a nick site with the highly conserved sequence CTTG ↓ ATA. This result indicates pTX1 possesses the same origin for RC replication. Around the conserved DSO sequence, two inverted repeat clusters are predicted to form stable stem-loop structures. The nick site is located next to the first stem-loop structure (Fig. 2).

Identification of a putative SSO of replication

The replication of the lagging strand of RC plasmids initiates from their SSO which is generally located at a short distance upstream of the DSO (Gruss *et al.*, 1987; Khan, 1997). The

		Nick site																													
pTX1	850-	C	C	T	C	C	T	T	A	-	C	T	T	G	A	T	A	C	-	A	T	A	G	A	A	C	A	A	-	877	
pPP8-1	1240-	G	C	T	C	T	T	T	A	C	T	T	G	A	T	A	C	-	A	T	A	G	A	A	A	C	A	A	-	1268	
pC194	1902-	C	T	T	T	C	T	T	A	T	C	T	T	G	A	T	A	A	T	A	A	G	G	G	T	A	A	C	T	-	1931
pUB110	242-	C	T	T	T	C	T	T	A	T	C	T	T	G	A	T	A	C	-	A	T	A	T	A	G	A	A	A	T	-	269
pBAA1	818-	T	T	T	T	C	T	T	A	T	C	T	T	G	A	T	A	C	T	A	T	A	T	A	G	A	A	A	C	-	846
pLP1	2048-	T	C	T	T	C	T	T	A	T	C	T	T	G	A	T	A	C	T	A	T	T	A	G	C	A	A	C	A	-	2077
pCB101	1478-	C	T	T	T	C	T	-	A	T	C	T	T	G	A	T	A	-	T	A	T	A	T	A	T	A	T	T	A	-	1505
pKYM	434-	T	A	T	A	C	T	T	A	A	G	G	-	G	A	T	A	-	A	A	T	G	G	C	G	G	A	T	A	-	460
φX174	4292-	C	T	C	C	C	C	C	A	A	C	T	T	G	A	T	A	T	T	A	A	T	A	A	C	A	C	T	A	-	4321

Fig. 4. Sequence alignment of DSOs from the pC194/pUB110 family. The nick site for the replication initiation is indicated. Nucleotides common to pTX1 are boxed and highly conserved region is highlighted in grey.

SSO functions only when they have been exposed in the form of ssDNA, and therefore replication of the lagging strand of RC plasmids does not start until the leading strand has been almost fully synthesized. There are several types of SSOs, such as *SSO_A*, *SSO_U*, *SSO_T*, and *SSO_W* based on their secondary structures (Khan, 1997). However, unlike the DSOs, their sequences are not homologous even in the same family. In addition, blocks of nucleotides conserved in hitherto known SSOs, as shown by Kramer *et al.* (1999), could not be identified in pTX1 (Kramer *et al.*, 1999). At position 2,093–2,261 of pTX1, three inverted repeats (IR-I–III) with a competence to form stem-loop structures may represent the SSO. Interestingly, the SSO of pTX1 is located quite far from DSO (Fig. 2).

Discussion

Autonomous replication and stable inheritance are the key properties of all plasmids. Plasmid copy number and size tend to be inversely related in all bacteria, and *Pseudomonas* species are no exception (Rehm, 2008). Most attention has been focused on the large, low-copy-number plasmids because they encode genes with specific function. In contrast, small, high-copy-number plasmids with genes of unknown function (cryptic) have been simply reported and studied, particularly with a view to vector development (Rehm, 2008). The role of these cryptic small plasmids in nature should be unveiled.

In this study, we report characterization of the small (2.2 kb), high-copy-number pTX1 plasmid isolated from *P. nitroreducens* TX1. Nucleotide BLAST queries revealed 76% coverage with pPP8-1 plasmid (2.5 kb) over the entire plasmid sequence. By analogy with the pC194/pUB110 plasmid family and ϕ X174, the strictly conserved sequence CTTGATA carrying the indicated nick site within the putative DSO was identified in pTX1, along with highly conserved amino acid residues (Lys-Tyr) in the catalytic domain of the predicted Rep_{pTX1} polypeptide. Both amino acid residues were previously shown to be instrumental in the nicking process and in circularization of single strand intermediates for RC replication. Though the predicted Rep_{pTX1} protein of pTX1 is only 12–22% similar to the corresponding proteins of the pC194/pUB110 plasmid family, three highly conserved motifs were detected in the pTX1 Rep protein. The motif I region LLTLTVKN is suggested to recognize the DSO (Ilyina and Koonin, 1992). The metal binding (HPHCHMILL: motif II with the conserved His residues) and catalytic (VFKYALKFSD: motif III) motifs were found in pTX1. Motif III included the putative active Tyr residue which conveys a nicking-closing activity necessary for RC replication (Ilyina and Koonin, 1992). SSO of pTX1 was also predicted based on

the extensive secondary structures (three inverted repeats) (Fig. 2).

Almost all of the plasmids hitherto described for *Pseudomonas* are considered to replicate bidirectionally. Only two studies reported the presence of RC plasmids in genus *Pseudomonas* so far. The first RC plasmid was isolated from *P. putida* P8. The predicted Rep protein and the putative replication origins place the replication system firmly in the pC194/pUB110 family of RC plasmids (Holtwick *et al.*, 2001). The other RC plasmids, p47L and p47S of *Pseudomonas* sp. strain S-47 have been reported (Chae *et al.*, 2005). Based on the Rep protein sequence comparison, p47L falls into the pIJ101/pJV1 family whereas p47S defines a new family of RC plasmid. Considering our results based on *in silico* analysis, it is concluded that the plasmid pTX1 in *P. nitroreducens* TX1 belongs to a new type of extrachromosomal element with RC replication in genus *Pseudomonas*.

적 요

Pseudomonas nitroreducens TX1은 대만의 벼를 재배하는 논에 배수구에서 분리된 세균이다. 이 균주는 알킬페놀 폴리에톡실레이트와 같은 비이온성 계면활성제를 고농도에서도 탄소 원료로 이용할 수 있다. 본 연구에서는 TX1 균주에서 분리된 새로운 플라스미드 pTX1의 특성을 조사하였다. 크기는 2,286 bp, GC 함량은 63.3%, 암호화된 유전자로는 Rep_{pTX1}과 기능이 밝혀지지 않은 ORF1과 ORF2가 동정되었다. Rep_{pTX1}은 롤링-서클 기작에 의해 복제되는 그람 양성 세균에서 주로 발견되는 pC194/pUB110 플라스미드 계열에 속하는 DNA 복제 효소임을 알 수 있었다. 또한 세포마다 약 150개의 플라스미드가 존재함을 규명하였다. 플라스미드에 존재하는 유전자 지도와 유사 플라스미드와의 핵산과 아미노산 서열비교를 통해 pTX1은 슈도모나스 세균에서는 흔히 발견되지 않는 롤링-서클 기작에 의해 복제된다는 것을 확인할 수 있었다.

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