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Isolation and Characterization of a Rolling-Circle-Type Plasmid from *Rhodococcus erythropolis* and Application of the Plasmid to Multiple-Recombinant-Protein Expression

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We isolated, sequenced, and characterized the cryptic plasmid pRE8424 from *Rhodococcus erythropolis* DSM8424. Plasmid pRE8424 is a 5,987-bp circular plasmid; it carries six open reading frames and also contains *cis*-acting elements, specifically a single-stranded origin and a double-stranded origin, which are characteristic of rolling-circle-replication plasmids. Experiments with pRE8424 derivatives carrying a mutated single-stranded origin sequence showed that single-stranded DNA intermediates accumulated in the cells because of inefficient conversion from single-stranded DNA to double-stranded DNA. This result indicates that pRE8424 belongs to the pIJ101/pJV1 family of rolling-circle-replication plasmids. Expression vectors that are functional in several *Rhodococcus* species were constructed by use of the replication origin from pRE8424. We previously reported a cryptic plasmid, pRE2895, from *R. erythropolis*, which may replicate by a θ-type mechanism, like ColE2 plasmids. The new expression vectors originating from pRE8424 were compatible with those derived from pRE2895. Coexpression experiments with these compatible expression vectors indicated that the plasmids are suitable for the simultaneous expression of multiple recombinant proteins.

Rhodococcus erythropolis is a gram-positive, high-G+C-content bacterium (*Actinobacteria*) that can grow at temperatures ranging from 4 to 35°C (39). Because many *Rhodococcus* strains have broad metabolic diversity and an array of unique enzymatic capabilities, they are of interest for pharmaceutical, environmental, chemical, and energy studies as well as for applications in the desulfurization of fossil fuels (25) and the industrial production of acrylamide (21). Several researchers have developed various genetic tools to analyze *Rhodococcus* (9), including *Escherichia coli-Rhodococcus* shuttle vectors (5) and a gene disruption system (38).

We have developed inducible expression vectors (pTip vectors) that work in several *Rhodococcus* species (26). The pTip vectors are tightly regulated expression vectors containing a *tipA* promoter (P_{tipA}) (4, 14), from which protein expression is induced by the antibiotic reagent thiostrepton (26). In our previous report, we showed that pTip vectors mediate heterologous and homologous protein expression, as they contain multiple cloning sites for 11 restriction enzyme sites and a hexahistidine (six-His) tag sequence and they work over a wide temperature range, from 4 to 35°C. The expression yields of recombinant proteins can be up to 10 mg per liter of *R. erythropolis* culture. In addition, some proteins that could not be expressed in *E. coli* were successfully expressed in *R. erythropolis*.

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The pTip vectors carry the *repAB* operon of the cryptic plasmid pRE2895, which is necessary for the autonomous replication of the plasmids in *Rhodococcus* cells. The *repAB* operon encodes the replication proteins RepA and RepB, which are characteristic of pAL5000-type plasmids (26, 35). The replication mechanisms of pAL5000 and pRE2895 are unknown, but RepA proteins of pAL5000 and pRE2895 are similar to Rep proteins of ColE2 plasmids (13), suggesting that they may replicate by a θ-type mechanism (35).

The *E. coli-Rhodococcus* shuttle vectors established so far, including the pTip vectors, have not been investigated in detail with regard to their replication mechanism. Few reports have addressed their cotransformation into *Rhodococcus*. In general, bacteria cannot be transformed with two different plasmids with the same replication origin because of plasmid incompatibility (27).

We report here the isolation and characterization of a new cryptic plasmid, pRE8424, from *R. erythropolis* DSM8424 and show that it replicates via a rolling-circle-type mechanism. Furthermore, we introduce new pTip vectors that are thiostrepton-inducible expression vectors and pNit vectors that are constitutive expression vectors. We succeeded in the stable cotransformation of *Rhodococcus* cells with two different plasmids without causing plasmid incompatibility. Moreover, we were able to coexpress two reporter proteins by using two different autonomous replication origins from pRE2895 and pRE8424.

MATERIALS AND METHODS

Strains, plasmids, oligonucleotides, and standard genetic manipulations. Tables 1 and 2 show all of the plasmids and bacterial strains used for this study. Plasmids were constructed by standard genetic manipulations (31). The trans-

TABLE 1. Plasmids used for this study

Plasmid	Characteristics[a]
pTip vectors	
pTip-QT1	P_{tipA} Tet^r *repAB* (pRE2895), MCS type 1
pTip-QT2	P_{tipA} Tet^r *repAB* (pRE2895), MCS type 2
pTip-RT1	P_{tipA} Tet^r *rep* (pRE8424), MCS type 1
pTip-RT2	P_{tipA} Tet^r *rep* (pRE8424), MCS type 2
pTip-QC1	P_{tipA} Chl^r *repAB* (pRE2895), MCS type 1
pTip-QC2	P_{tipA} Chl^r *repAB* (pRE2895), MCS type 2
pTip-RC1	P_{tipA} Chl^r *rep* (pRE8424), MCS type 1
pTip-RC2	P_{tipA} Chl^r *rep* (pRE8424), MCS type 2
pNit vectors	
pNit-QT1	P_{nit} Tet^r *repAB* (pRE2895), MCS type 1
pNit-QT2	P_{nit} Tet^r *repAB* (pRE2895), MCS type 2
pNit-RT1	P_{nit} Tet^r *rep* (pRE8424), MCS type 1
pNit-RT2	P_{nit} Tet^r *rep* (pRE8424), MCS type 2
pNit-QC1	P_{nit} Chl^r *repAB* (pRE2895), MCS type 1
pNit-QC2	P_{nit} Chl^r *repAB* (pRE2895), MCS type 2
pNit-RC1	P_{nit} Chl^r *rep* (pRE8424), MCS type 1
pNit-RC2	P_{nit} Chl^r *rep* (pRE8424), MCS type 2
PIP expression vectors	
pHN380	Six-His–PIP in MCS of pTip-RC1
pHN389	Six-His–PIP in MCS of pNit-RC1
pHN409	Six-His–PIP in MCS of pNit-QC1
GFP expression vectors	
pHN425	Six-His–GFP in MCS of pNit-QT1
pHN426	Six-His–GFP in MCS of pNit-RT1
Vectors for identification of DSO and SSO of pRE8424	
pHN317	PCR fragment of pRE8424 (nucleotides 3283 to 5987 and 1 to 400) in KpnI and XbaI sites of pHN267
pHN345	TAGCGG in IR I of pHN317 was changed to CCATGG by site-directed mutagenesis
pHN362	TAGCGG in IR II of pHN317 was changed to CCATGG by site-directed mutagenesis
pHN363	TAGCGG in IR II of pHN345 was changed to CCATGG by site-directed mutagenesis
pHN322	PCR fragment of pRE8424 (nucleotides 3418 to 5987 and 1 to 400) in KpnI and XbaI sites of pHN267
pHN343	Deletion derivatives of pHN317; digested with KpnI and SacII, blunt ended, and self-ligated
pHN344	Deletion derivatives of pHN317; digested with SalI and XbaI, blunt ended, and self-ligated
pHN324	PCR fragment of pRE8424 (nucleotides 3283 to 5507) in KpnI and XbaI sites of pHN267

[a] MCS, multiple cloning site.

formation of *Rhodococcus* strains and the isolation of plasmids from *R. erythropolis* were performed by a previously described method (26). *Rhodococcus* strains and *E. coli* strains were routinely cultured in Luria broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 0.5% NaCl) in the presence or absence of appropriate antibiotics. The antibiotics used to select transformants in the culture media were tetracycline (8 μg/ml in liquid medium and 20 μg/ml in solid medium), chloramphenicol (34 μg/ml), kanamycin (200 μg/ml for *R. erythropolis* and 10 μg/ml for *E. coli*), and ampicillin (50 μg/ml). Genomic DNAs from *Rhodococcus* and *Streptomyces* species were isolated by a previously described method (20). Genomic DNA from *E. coli* was isolated with an RNA/DNA mini kit (Qiagen, Inc.). PCRs were performed with *Pfu* turbo polymerase (Stratagene). T4 polynucleotide kinase (Toyobo Co., Ltd.) was used to phosphorylate the DNA fragments or the oligonucleotides.

Construction of plasmids. A PCR was performed with two primers (GTTGT ACAAGCATGGGGACTCGCCGC and GAAGCTGACCAAGTTCTC) and with pRE8424 as a template. The 5′ ends of the amplified fragment (nucleotides 3845 to 5849) were phosphorylated, the resulting fragment was cloned into the HincII site of pBluescript II SK(+) (Stratagene), and subsequently the BamHI site in the *rep* gene (*orf6*) (Fig. 1A) was eliminated by site-directed mutagenesis, yielding pHN372.

A 1.8-kb fragment excised from pHN346 (26) by the use of XbaI and SpeI was cloned into the XbaI site of pHN154 (26), yielding pHN347. A 1.1-kb fragment excised from pHN171 (26) by the use of BsrGI and SpeI was cloned into the BsrGI and SpeI sites of pHN347, yielding pHN348. A PCR was performed with two primers (AACGTTGTCTTTATGTTGGATC and AATGTACAAGTTAA CGACCGCGCGGGTCCCGGACG) and with pHN171 as a template to amplify a 0.2-kb fragment containing the 5′ part of the thiostrepton resistance gene. This fragment was digested with BsrGI and ClaI, and the resulting fragment was cloned into the BsrGI and ClaI sites of pHN171 and pHN348, yielding pHN357 and pHN358, respectively. A 2.0-kb fragment containing the minimal region for the autonomous replication of pRE8424, excised from pHN372 by the use of BsrGI and HpaI, was cloned into the BsrGI and HpaI sites of pHN357 and pHN358, yielding pHN379 and pHN380, respectively.

A double-stranded DNA (dsDNA) fragment containing unique restriction enzyme sites and six continuous histidine codons to translate a six-His-tagged fusion protein was prepared by annealing two oligonucleotides. The annealed double-stranded DNA fragment was cloned into the NcoI and SpeI sites of pHN379 and pHN380, yielding pTip-RT1 and pTip-RC1, respectively (Table 1). The annealed dsDNA and a 0.2-kb fragment excised from pTip-LNH2 (26) by the use of BsrGI and NdeI were simultaneously cloned into the BsrGI and SpeI

TABLE 2. Bacterial strains used for this study

Species	Strain	Source	Application
R. erythropolis	JCM2895	Japan Collection of Microorganisms	Source of pRE2895
	DSM8424	German Collection of Microorganisms and Cell Cultures	Source of pRE8424
	JCM2893	Japan Collection of Microorganisms	Source of pRE2893
	JCM2894	Japan Collection of Microorganisms	Source of pRE2894
	DSM43200	German Collection of Microorganisms and Cell Cultures	Source of pRE43200
	JCM3201	Japan Collection of Microorganisms	Host strain to express recombinant proteins
R. fascians	JCM10002	Japan Collection of Microorganisms	Host strain to express recombinant proteins
R. opacus	DSM44193	German Collection of Microorganisms and Cell Cultures	Host strain to express recombinant proteins
R. ruber	JCM3205	Japan Collection of Microorganisms	Host strain to express recombinant proteins
R. rhodochrous	JCM3202	Japan Collection of Microorganisms	Host strain to express recombinant proteins
E. coli	DH5α		General cloning
	ER2508	New England BioLabs Inc.	Source of Kan^r gene
	GM2163	New England BioLabs Inc.	Methylation-negative strain

sites of pHN379 and pHN380, yielding pTip-RT2 and pTip-RC2, respectively (Table 1). A 0.3-kb fragment excised from pTip-RT1 by the use of BsrGI and SpeI was cloned into the BsrGI and SpeI sites of pHN171 and pHN348, yielding pTip-QT1 and pTip-QC1, respectively. A 0.3-kb fragment excised from pTip-RT2 by the use of BsrGI and SpeI was cloned into the BsrGI and SpeI sites of pHN171 and pHN348, yielding pTip-QT2 and pTip-QC2, respectively.

An inverse PCR (28) was performed with two divergent primers (TGACGC CGTCCATTATACCTCCTCACGTG and GAGAAGGGAGCGGCCATGGC) and with pHN150u (26) as a template. The amplified fragment was circularized by self-ligation, yielding pHN231.

A 1.6-kb fragment containing the tetracycline resistance gene was PCR amplified from pTip-NH1 (26) by the use of two primers (TTTGTTAACTAGAG TAACGGGCTACTCCG and AAGGTACCTCAACGACAGGAGCACGA TC). The amplified fragment was digested and cloned into the HpaI and KpnI sites of pHN379, yielding pHN381. Another antibiotic resistance gene, the chloramphenicol resistance gene (1.8 kb), was PCR amplified from pHN346 by the use of two primers (ACTGTTAACGCATCCGAAACCTCCACCCCACTC and GC TGTAGTGGAACTCACCGAGCAC). The amplified fragment was digested and cloned into the HpaI and KpnI sites of pHN380, yielding pHN382.

The promoter region and the genes essential for plasmid replication were prepared as follows. The P_{nit}-containing fragment (0.2 kb) was PCR amplified from pHN231 by the use of two primers (CGTGTACATATCGAGGCGGGC TCCCA and TTTCTAGACGCCGTCCATTATACCTCCTCACGTG). This fragment was digested and cloned into the BsrGI and XbaI sites of pHN381 and pHN382, yielding pHN385 and pHN389, respectively. For the introduction of the *repAB* operon of pRE2895 into pHN385 and pHN389, a PCR was performed with two primers (AAAGTTAACGAGAGTTGGCCGTTGCTC and GCTGT ACACCCGAGAAGCTCCCAGCG) and with pHN171 as a template. A 1.9-kb fragment was digested and cloned into the BsrGI and HpaI sites of pHN385 and pHN389, yielding pHN407 and pHN409, respectively.

A 2.2-kb fragment excised from pTip-RT1 by the use of NcoI and KpnI was cloned into the NcoI and KpnI sites of pHN385, pHN389, pHN407, and pHN409, yielding pNit-RT1, pNit-RC1, pNit-QT1, and pNit-QC1, respectively. A PCR was performed with two primers (CGTGTACATATCGAGGCGGGC TCCCA and AACATATGTATATCTCCTTCTTAAAGTTAAAC) and with pHN385 as a template to amplify a 0.2-kb fragment. The amplified fragment, digested with BsrGI and NdeI, and a 2.0-kb fragment excised from pTip-RT2 by the use of NdeI and KpnI were simultaneously cloned into the BsrGI and KpnI sites of pHN385, pHN389, pHN407, and pHN409, yielding pNit-RT2, pNit-RC2, pNit-QT2, and pNit-QC2, respectively.

Recombinant protein expression and purification and measurements of proline iminopeptidase (PIP) activity. The gene encoding green fluorescent protein (GFP) was amplified by a PCR, with pHN187 (26) as a template. The amplified DNA fragment was subcloned into the NcoI and BamHI sites of pNit-QT1 and pNit-RT1 (Table 1). The NcoI site within the GFP coding sequence was eliminated by site-directed mutagenesis.

When recombinant proteins were expressed by inducible expression vectors in *R. erythropolis* at 30°C, the cells were grown to an optical density at 600 nm (OD_{600}) of 0.6 before thiostrepton (final concentration, 1 μg/ml) was added, and the cells were then cultured for an additional 16 h. When recombinant proteins were expressed by constitutive expression vectors, the cells were grown to an OD_{600} of 2.0. The purification of recombinant proteins containing a six-His tag and measurements of PIP peptidase activity (36) were performed by previously described methods (26).

Detection of ssDNA. For the detection of single-stranded DNA (ssDNA), total DNAs from the *Rhodococcus* strains used in Southern hybridization experiments were prepared by the method described by Fernandez-Gonzalez et al. (8), with slight modifications as follows. Luria broth was used instead of tryptic soy broth; also, 250 μl of lysis buffer was used. When necessary, rifampin was added to 100 μg/ml and the cultures were incubated for another hour before DNA extraction. S1 nuclease digestion and subsequent electrophoresis were also performed according to the methods described by Fernandez-Gonzalez et al. (8). S1 nuclease was purchased from Takara Shuzo Co. Ltd. DNAs separated in agarose gels were transferred to Hybond N^+ membranes (Amersham Biosciences Corp.) without prior denaturation with alkali. Southern hybridization and signal detection were performed by use of the ECF random-prime labeling and detection system (Amersham Biosciences Corp.) and an FX Pro molecular imager (Bio-Rad Laboratories, Inc.). We used 1 μg of probe to label the dsDNA probe. Synthetic oligonucleotide probes were 5′ end labeled with fluorescein isothiocyanate (Hokkaido System Science Co., Ltd.), and 1 nmol of each probe was used. The sequence of the oligonucleotide probe Fwd. was 5′ TCACGGATGCTCAGAT TGTCGAACAGGAAG 3′ and that of the oligonucleotide probe Rev. was 5′ CTTCCTGTTCGACAATCTGAGCATCCGTGA 3′.

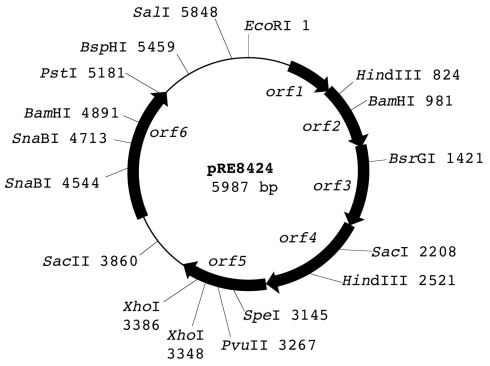
Estimation of plasmid copy number. The genome size of *R. erythropolis* JCM3201 needed to be determined to estimate plasmid copy numbers by the method of Projan et al. (29), but the genome size remains unknown. However, the genome size of *R. erythropolis* strain RG1 has been estimated to be 6 Mbp (37), and *R. erythropolis* strain RG1 is a derivative of *R. erythropolis* ATCC 4277, which is in turn an equivalent strain to *R. erythropolis* JCM3201. We therefore assumed that the genome size of *R. erythropolis* JCM3201 is also 6 Mbp. We used an FX Pro molecular imager to estimate DNA band intensities.

Nucleotide sequence accession numbers. The sequence data for pRE8424 and pHN267 have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB127588 and AB127589, respectively. The sequence data for 16 pTip and pNit vectors have also been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB127590 (pTip-QC1), AB127591 (pTip-QC2), AB127592 (pTip-QT1), AB127593 (pTip-QT2), AB127594 (pTip-RC1), AB127595 (pTip-RC2), AB127596 (pTip-RT1), AB127597 (pTip-RT2), AB127598 (pNit-QC1), AB127599 (pNit-QC2), AB127600 (pNit-QT1), AB127601 (pNit-QT2), AB127602 (pNit-RC1), AB127603 (pNit-RC2), AB127604 (pNit-RT1), and AB127605 (pNit-RT2).

RESULTS

Detection of small endogenous plasmids in several *R. erythropolis* strains. We searched for endogenous small plasmids in several *R. erythropolis* strains. We found small cryptic circular plasmids in *R. erythropolis* JCM2893, *R. erythropolis* JCM2894,

(A)



(B)

		Motif IV		Motif I		Motif II		Motif III
Consensus		GLXXCGXXWXCPXC		XvtXTXRH		gXXgXXraXeXtXgXXnGwHXHXhXlX		laXYXXKXqX
pRE8424	68	GLRSCGKGWICPCC	26	MVTMTMRH	33	GCDGYVRAVEITHGK-NGWHVHVHALL	53	LAAYLTKIAS
pAP1	138	GLHTCGSVWACPVC	27	MLTLTQRH	33	GLVGYVRANEITHGK-HGWHVHSHVLI	67	IGNYVSKMQT
pBL1			76	MFVGTVRH	34	VEHTYSDYEVTDSWA-NGWHLHRNMLL	54	MATYLAKGMS
pJV1	38	GLVRCGRIWFCPEC	27	LVTFTARH	77	GYIGMVRAAEVTRSKKNGYHPHLNLLV	80	LIEYLTKNQD
pIJ101	20	GLMRCGRIWLCPVC	27	LVTFTARH	59	GYVGMIRATEVTVGQINGWHPHIHAIV	69	LAEYIAKTQD
pSN22	20	GLMRCGRIWLCPVC	27	LVTFTARH	59	GYVGMIRATEVTVGQINGWHPHIHAIV	69	LAEYIAKTQD
		** ** .*.** *		:..:* **		: . :.. ... .: .*:* * :.::		:. *::*

		C-terminal motif
Consensus		WXeyEXaXXgrRaiXWXrglr
pRE8424	276	WREFEFGSMGRRAIAWSRGLR
pAP1	365	WKEYEKASFGRRALTWSKGLR
pBL1	250	WREYEVGSKNLRS-SWSRGAK
pJV1	352	WAQYEEALAGRRAIEWTRGLR
pIJ101	288	WHEYERATRGRRAIEWTRYLR
pSN22	288	WHEYERATKGRRAIEWTRYLR
		* ::* . * * : * : ::

FIG. 1. Schematic map of pRE8424 and motif alignment of the Rep protein encoded by *orf6*. (A) Schematic map of pRE8424. Some restriction enzyme sites are shown, followed by their positions, in base pairs, from the zero point at the top of the map, arbitrarily defined by an EcoRI site. Closed arrows indicate the six ORFs. (B) Amino acid motifs of pRE8424 Rep protein. The sequences show the identification of the five motifs that are conserved in Rep proteins of RCR plasmids (2, 16). These motifs are aligned with homologous regions of Rep proteins from pRE8424, pAP1 (2), pBL1 (8), pJV1 (33), pIJ101 (18), and pSN22 (17). Numbers indicate the numbers of amino acids between motifs for each protein. Asterisks indicate perfectly conserved residues, whereas well-conserved residues are indicated by dots. Tyrosine residues involved in DNA linking are boxed.

R. erythropolis DSM43200, and *R. erythropolis* DSM8424, and these plasmids were designated pRE2893, pRE2894, pRE43200, and pRE8424, respectively.

A sequence analysis of pRE2893, pRE2894, and pRE43200 revealed that these plasmids are all almost identical to plasmid pRE2895, which we isolated previously from *R. erythropolis* JCM2895 (26). On the other hand, pRE8424 was quite different from pRE2895. pRE8424 carries six open reading frames (ORFs) (*orf1* to *orf6*; Fig. 1A). In the case of *orf1*, *orf2*, *orf3*, and *orf4*, stop codons of preceding ORFs overlapped with the start codons of subsequent ORFs, suggesting that their translation is coupled. Among these ORFs, only *orf4* and *orf6* encode proteins with significant similarities to known proteins. The *orf4* gene encodes a protein that shares a low sequence similarity with the DNA translocase of *Bacillus halodurans* and with FtsK-like proteins, which play a role in cell division (1). The *orf6* gene encodes a protein similar to the replicase protein (Rep) of rolling-circle-replication (RCR) plasmids (19) (Fig. 1B); pRE8424 Rep is most similar to the Rep protein of pAP1 from *Arcanobacterium pyogenes* (2). pAP1, pIJ101 from *Streptomyces lividans* (18), pJV1 from *Streptomyces phaeochromogenes* (33), pBL1 from *Brevibacterium lactofermentum* (8), and pSN22 from *Streptomyces nigrifaciens* (17) comprise the pIJ101/pJV1 family plasmids (19). An alignment of the amino acid sequences of the Rep proteins of all of these plasmids revealed five conserved motifs that are involved in RCR initiation (Fig. 1B) (2, 16). Motif III contains a conserved tyrosine residue that is inferred to be involved in covalent linkage to the 5′ end of the nicked ssDNA strand (16). These facts indicate that pRE2824 replicates by RCR and that it belongs to the pIJ101/pJV1 family.

Recently, a small cryptic plasmid, pAN12, was isolated from *R. erythropolis* (22). It apparently belongs to the pIJ101/pVJ1 family and its nucleotide sequence is highly similar to that of pRE8424 (91.7% identity).

Mode of replication of pRE8424 and localization of regions involved in its autonomous replication. RCR plasmids usually contain not only the gene encoding Rep but also *cis*-acting elements such as a double-stranded origin (DSO) and a single-stranded origin (SSO) that are essential for plasmid replication

(19). To identify and characterize the DSO and SSO regions of pRE8424, we used pHN267 (GenBank accession number AB127589), which carries a kanamycin resistance gene as a selection marker for *R. erythropolis*. Derivatives of pHN267 (Table 1; Fig. 2) were constructed by subcloning DNA fragments of pRE8424 into pHN267.

As shown in Fig. 2, the plasmid-free strain *R. erythropolis* JCM3201 was transformed successfully with pHN317, indicating that only the *rep* gene of the six ORFs is necessary for maintaining the plasmid in *R. erythropolis*. Experiments with deletion derivatives of pHN317 plasmids (pHN322, pHN343, pHN344, and pHN324) demonstrated that the region deleted from pHN324 (nucleotides 5514 to 5970 in the pRE8424 sequence) contains the essential function for plasmid replication. Homology searches revealed that the putative DSO-like sequence was found between nucleotides 5705 and 5734 on pRE8424. In this sequence, the GG dinucleotides, a putative nicking site for Rep (2), are conserved (Fig. 3A). Based on these results, we predicted that the identified sequence functions as the DSO of pRE8424.

The overall nucleotide sequences of SSOs of RCR plasmids are not generally well conserved. However, in all pIJ101/pJV1 family plasmids, SSOs contain inverted repeat sequences (IRs) and a conserved hexanucleotide sequence, TAGCGT, in the loop region of the stem-loop structure (2). As shown in Fig. 3B, two putative IR sequences, IR I and IR II, both of which contain a TAGCGG sequence in the loop region of the stem-loop structures, were found in pRE8424. When bacteria are transformed with RCR plasmids lacking SSO sequences, ssDNA intermediates frequently accumulate in the cell as a result of inefficient conversion from ssDNA to dsDNA (19). We investigated the accumulation of ssDNA intermediates in *R. erythropolis* cells to examine whether the IRs function as SSOs (Fig. 2). Total DNAs from *R. erythropolis* JCM3201 cells transformed with plasmids were extracted, treated or not treated with S1 nuclease, which degrades only ssDNA, and subjected to Southern hybridization with the StuI/BamHI fragment of pRE8424 (0.7 kb) as a probe (Fig. 2). As controls, total DNAs from *R. erythropolis* JCM3201 without plasmids and *R. erythropolis* DSM8424, which harbors pRE8424, were also subjected to Southern hybridization. Large quantities of ssDNA intermediates accumulated in cells transformed with pHN362 or pHN363, whereas ssDNA intermediates did not accumulate in cells transformed with pHN345 or pHN343 (Fig. 2 and 4A). This showed that the replacement of TAGCGG with CCATGG inactivated IR II and that only IR II functions as an SSO that is essential for plasmid replication (Fig. 2 and 4A). A rifampin treatment usually induces the accumulation of ssDNAs by blocking the action of RNA polymerase because lagging-strand synthesis of most RCR plasmids is dependent on cellular RNA polymerase (32). However, ssDNAs did not accumulate in *R. erythropolis* DSM8424 treated with rifampin, suggesting that the lagging-strand synthesis of pRE8424 may be independent of RNA polymerase. Southern hybridization was also performed with a sense (Fwd.) and an antisense (Rev.) oligonucleotide as probes (Fig. 2) (see Materials and Methods). A prominent band was observed only when oligonucleotide probe Rev. was used (Fig. 4B), indicating that the accumulated ssDNA intermediate of pRE8424 is the same strand found in the Rep-like plasmid of the pIJ101/pJV1 family.

Construction of inducible and constitutive expression vectors using part of pRE2895 and pRE8424. Our previous study reported new thiostrepton-inducible expression vectors named pTip vectors. pTip vectors carry a 1.9-kb region containing a putative replication origin and the *repAB* operon derived from pRE2895; pRE2895 is 5.4 kb long, and the 1.9-kb region has been shown to be sufficient for autonomous replication in *R. erythropolis* (26). We replaced the multiple cloning sites of pTip vectors (pTip-LNH1 and pTip-LNH1.2) with more convenient ones, yielding the new pTip vectors pTip-QT1, pTip-QT2, pTip-QC1, and pTip-QC2 (schematic maps and descriptions are shown in Fig. 5 and Table 1, respectively). Next, we replaced the 1.9-kb region of these pTip vectors with a 2.0-kb region of pRE8424 containing the *rep* gene, the DSO, and the SSO, yielding pTip-RT1, pTip-RT2, pTip-RC1, and pTip-RC2, respectively (Fig. 5 and Table 1).

The sequence of TATAAT is known as a consensus sequence of −10 regions of strong *E. coli* promoters (7). We therefore substituted TATAAT for the −10 sequence of the *tipA* promoter (CAGCGT) (Fig. 5B) and determined whether the protein expression level changed. In cells transformed with pHN380, a plasmid containing P_{tipA}, the expression of a reporter protein, PIP (36), was inducible by the use of thiostrepton (Fig. 6). However, in cells transformed with pHN389, a plasmid containing the modified promoter, the expression of the reporter protein was reproducibly observed in the absence of thiostrepton (Fig. 6). Moreover, the expression of the reporter protein was regulated in a TipAL-independent manner, indicating that the modified promoter is a constitutive promoter (Fig. 6). Therefore, we designated this constitutive promoter the *nit* (noninducible *tipA*) promoter (P_{nit}). Using P_{nit}, we constructed pNit vectors which express recombinant proteins constitutively (Fig. 5; also see Materials and Methods). The expression level from P_{nit} was always about half that from P_{tipA}, even when different reporters were used (data not shown).

We constructed eight inducible expression vectors (pTip vectors) and eight constitutive expression vectors (pNit vectors) that work in *R. erythropolis* (Fig. 5 and Table 1). Because the ribosome binding site (RBS) for phage *T7* gene 10 (LG10-RBS) leads to a higher expression level than the *tipA* RBS, it was introduced into all of the expression vectors (Fig. 5) (26).

Comparative analysis of fragments derived from pRE2895 and pRE8424 for autonomous replication. We compared the transformation efficiencies of *Rhodococcus* cells with pNit-QC2 (carries the replication origin from pRE2895) and pNit-RC2 (carries the replication origin from pRE8424). The transformation efficiencies of *R. erythropolis* cells with pNit-QC2 or pNit-RC2 were similar (3.8×10^5 and 2.8×10^5 CFU/μg of DNA, respectively). On the other hand, the transformation efficiency of *Rhodococcus fascians* cells with pNit-QC2 (8.2×10^2 CFU/μg of DNA) was slightly higher than that with pNit-RC2 (4.0×10^2 CFU/μg of DNA), and the transformation efficiency of *Rhodococcus opacus* cells with pNit-QC2 (1.6×10^4 CFU/μg of DNA) was obviously higher than that with pNit-RC2 (5.2×10^2 CFU/μg of DNA). For both pNit-QC2 and pNit-RC2, no transformants of *Rhodococcus rhodochrous* or *Rhodococcus ruber* cells were observed. These results indi-

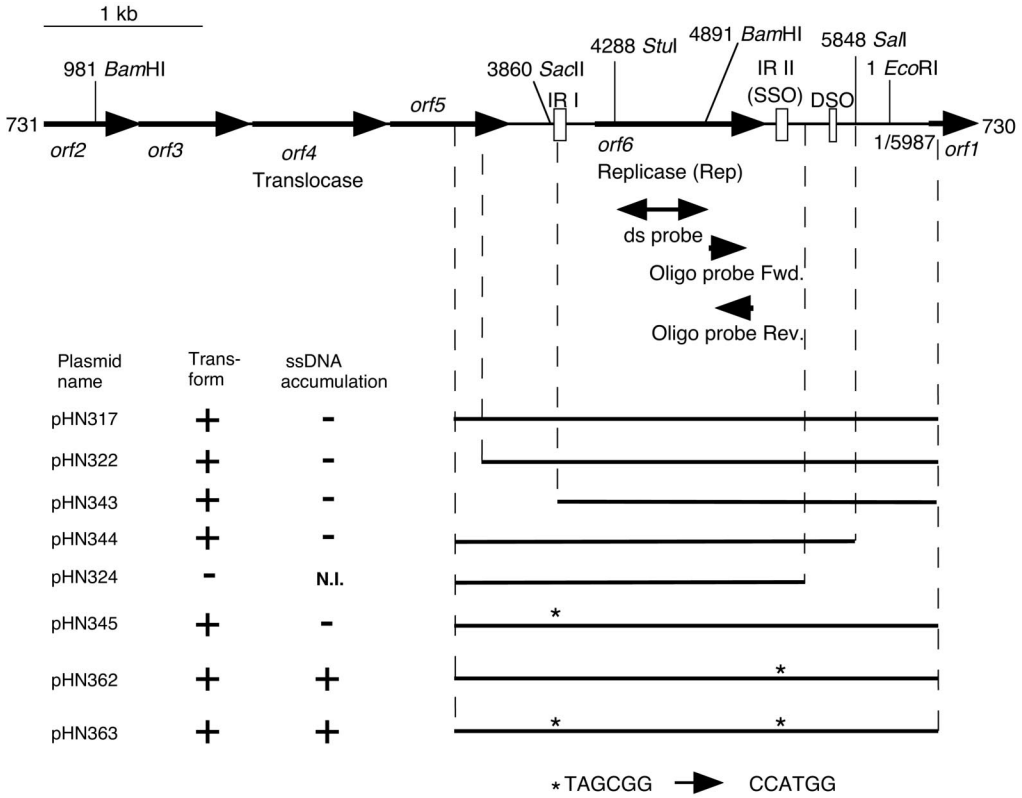


FIG. 2. Identification of DSO and SSO. A Derived map of pRE8424, its transformation ability for *R. erythropolis* JCM3201, and the results of a ssDNA accumulation experiment (see Fig. 4A) are shown. The derived plasmids of pRE8424 resulting from subcloning into plasmid pHN267 are shown with lines. The transformation ability of each plasmid for *R. erythropolis* JCM3201 is indicated (+ or −). +, plasmids that accumulated in *R. erythropolis* JCM3201 in a ssDNA form (see Fig. 4A); N.I., no information. Asterisks indicate the introduction of mutations in the TAGCGG sequence (see Fig. 3B). The positions of double-stranded probes (ds probe) and two oligonucleotide probes (Oligo probe Fwd. and Oligo probe Rev.) used for Southern hybridization (see Fig. 4) are indicated by arrows. The IRs and DSO are indicated (see Fig. 3A). Only IR II functions as an SSO (see Fig. 4A.).

cate that the replication origin from pRE8424 has a narrower host range than that of pIJ101/pJV1 family members (19). The estimated copy numbers of pNit-QC2 and pNit-RC2 were 47 ± 5/cell and 64 ± 5/cell, respectively (see Materials and Methods).

The expression levels of the reporter protein in different *Rhodococcus* species were determined. The PIP-encoding gene was cloned into pNit vectors, yielding pHN409 (carries the replication origin from pRE2895) and pHN389 (carries the replication origin from pRE8424) (Table 1). *R. erythropolis*, *R. fascians*, and *R. opacus* were transformed with either pHN409 or pHN389. The cells were inoculated at 30°C and their PIP activities were measured. In all *Rhodococcus* hosts, cells transformed with pHN409 showed comparable or slightly higher PIP activities than cells transformed with pHN389 (data not shown). PIP activities in *R. fascians* and *R. opacus* cells were lower than that in *R. erythropolis* (data not shown), but it remains unclear whether the differences in the expression levels were due to differences in gene expression, the stabilities of the plasmids, or the copy numbers of the plasmids. The expression levels of PIP in cells transformed with pTip vectors were similar to that reported previously (26).

Plasmid compatibility analysis. We next determined plasmid compatibilities by the serial transformation of *R. erythropolis* with two plasmids carrying different or the same replication origins (Table 3). First, transformation was carried out with either pNit-QC2 or pNit-RC2 and each transformant was selected on chloramphenicol-containing plates; isolated transformants were transformed subsequently with either pNit-QT2 or pNit-RT2. Each transformant was selected on a tetracycline-containing plate. When the second transformations were carried out with plasmids containing the same replication origin as the resident plasmids, the CFU per 1 μg of DNA were reduced about 2 orders of magnitude. On the other hand, transformation efficiencies were not significantly reduced when second transformations were carried out with plasmids containing different replication origins from the resident plasmids. We picked 200 independent colonies from each tetracycline-containing plate after the second transformations and plated them onto chloramphenicol- or tetracycline- and chloramphenicol-containing plates to examine the maintenance of plasmids (Table 3). When second transformations were carried out with plasmids containing the same replication origin as the resident plasmids, the resident plasmids were frequently lost, whereas resident plasmids were not lost when two plasmids with different replication origins were used. Moreover, when two plasmids with the same replication origin were used, colony numbers on tetracycline- and chloramphenicol-containing

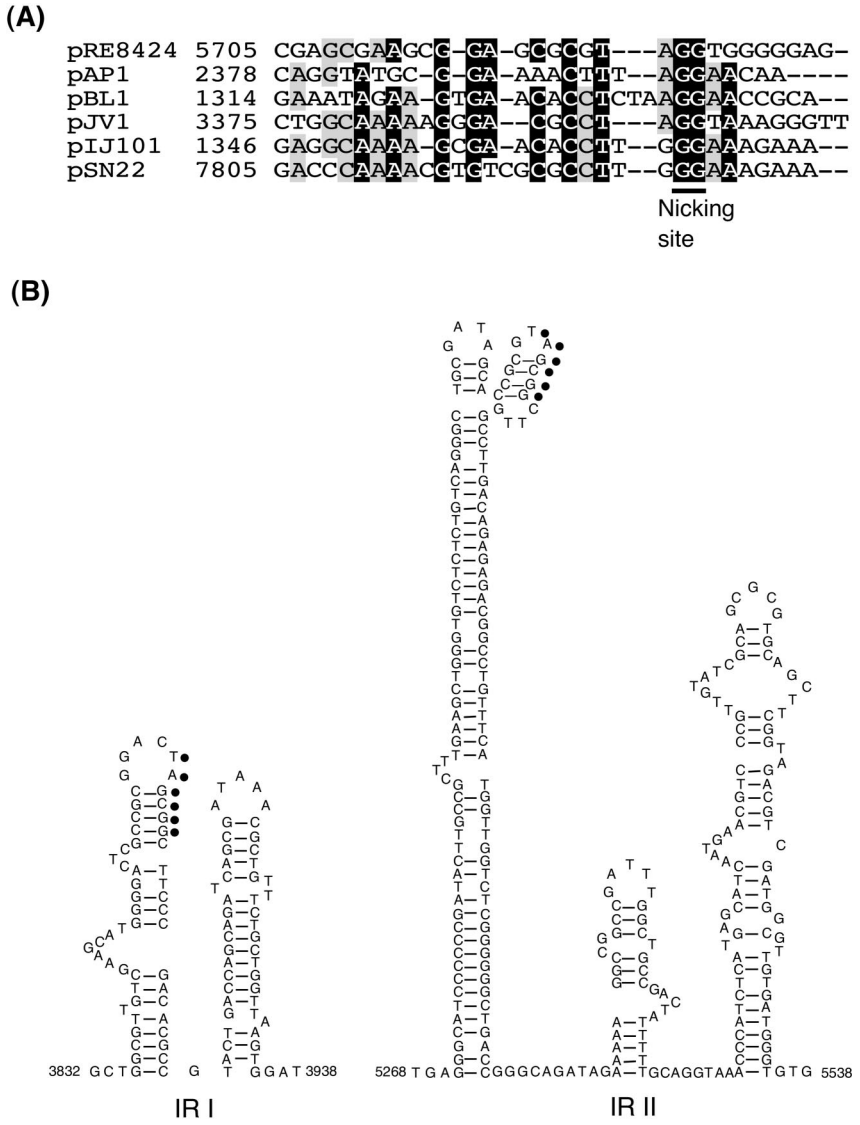


FIG. 3. DSO and SSO sequences of pRE8424. (A) Alignment of nucleotide sequence of the putative DSO of pRE8424 with those of the pIJ101/pJV1 family. The nick sites that are conserved in all of the RCR plasmids as GG dinucleotides are underlined. Positions with >80% identity are indicated with a black background and positions with >60% identity are indicated with a dotted background. (B) Sequences and predicted secondary structures of IR I and IR II found in pRE8424. The secondary structures of the two IRs were predicted with the mfold program, version 3.0 (Michael Zuker, Washington University, St. Louis, Mo. [http://www.bioinfo.rpi.edu/applications/mfold/old/dna/form1.cgi]). The sequence TAGCGG, which is similar to the SSO consensus sequence TAGCGT defined by Zaman et al. (40), is labeled with closed circles.

plates were far smaller than those for plasmids with different replication origins. These results indicate that plasmid derivatives of pRE2895 and pRE8424 are fully compatible in *R. erythropolis*.

Coexpression of recombinant proteins in *R. erythropolis* cells by cotransformation with expression vectors. The development of fully compatible expression vectors encouraged us to coexpress recombinant proteins in a single *Rhodococcus* strain by the cotransformation of two expression vectors. We tried to coexpress six-His–PIP and six-His–GFP in *R. erythropolis* JCM3201. Cells were cotransformed with pHN425 and pHN389 (Table 1) or with pHN426 and pHN409 (Table 1). As controls, cells were also transformed with either pHN425, pHN426, pHN389, or pHN409 alone. Proteins were purified by Ni-nitrilotriacetic acid affinity chromatography.

As shown in Fig. 7, we successfully expressed and purified recombinant PIP and GFP simultaneously in a single cell type without reducing the expression levels compared to those achieved by the separate expression of each protein. The amounts of isolated proteins were about 1 mg of GFP and about 3 mg of PIP per 1 liter of *R. erythropolis* culture from the P_{nit} constitutive promoter.

R. erythropolis JCM3201 cotransformed with pHN425 and pHN409 grew poorly in liquid medium containing tetracycline and chloramphenicol, and the expression levels of PIP and GFP in those transformants were not consistent (data not

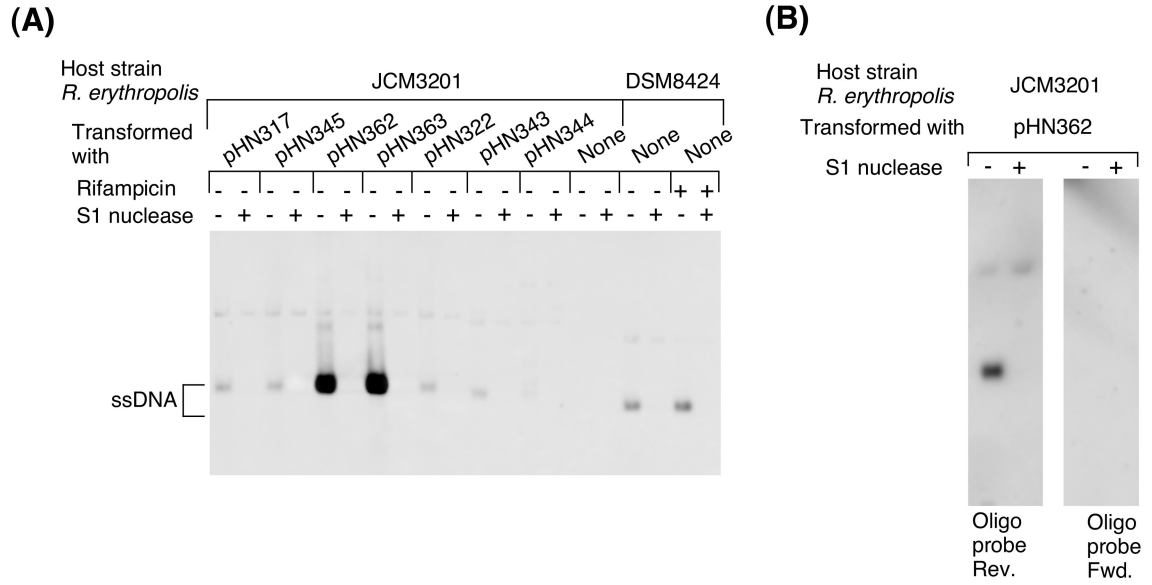


FIG. 4. Detection of ssDNA intermediates of pRE8424 derivatives by Southern hybridization. (A) Southern hybridization with a double-stranded probe (see Fig. 2). Total DNAs from the indicated *R. erythropolis* strains transformed with the indicated plasmids or from the indicated nontransformed cells (None) were extracted and treated or not treated with S1 nuclease (+ or −, respectively). The samples were subjected to Southern hybridization with a double-stranded probe. In some cases, rifampin was added to block the action of cellular RNA polymerase. (B) Southern hybridization with oligonucleotide probes (see Fig. 2). Total DNAs from *R. erythropolis* JCM3201 transformed with pHN362 were subjected to Southern hybridization with two different oligonucleotide probes (see Fig. 2).

shown). The cells transformed with pHN426 and pHN389 hardly grew in the doubly selective medium, suggesting that replication via single-stranded intermediates is unstable.

DISCUSSION

Herein, we describe the new cryptic plasmid pRE8424 from *R. erythropolis* and its application for the expression of multiple proteins. Experimental results indicate clearly that the pRE8424 replicates by a rolling-circle mechanism (Fig. 4). This replication mechanism differs markedly from that of pRE2895, another cryptic plasmid previously isolated from *R. erythropolis*, which may replicate by a θ-type mechanism, like ColE2 plasmids (13). In *Rhodococcus*, several endogenous plasmids other than pRE2895 and pRE8424 have been identified. pFAJ2600, isolated from *R. erythropolis* NI86/21 (5), has a high level of similarity to pRE2895, and pMVS300, isolated from *Rhodococcus* sp. strain H13-A (34), has a unique sequence. Very recently, plasmid pAN12, which shows high nucleotide sequence similarity to pRE8424 (91.7% identity), was isolated from *R. erythropolis* strain AN12 (22). In that report, the authors identified a putative DSO sequence in pAN12 by multiple sequence alignments of DSO sites. Based on the results of our experiments (Fig. 2), the DSO sequence they defined for pAN12 differs from the one defined herein. The putative DSO sequence in pAN12 is not well conserved in pRE8424, but the DSO sequence we identified in pRE8424 was conserved perfectly in pAN12. Furthermore, in pRE8424, the region similar to the putative DSO of pAN12 (nucleotides 229 to 252 of pRE8424) was dispensable (Fig. 2). These results strongly suggest that the actual DSO in pAN12 is the region similar to the putative DSO of pRE8424. The plasmid pRE8424 is the first experimentally characterized RCR plasmid among several RCR plasmids isolated from *Rhodococcus* and *Rhodococcus*-related genera such as *Corynebacterium* (23, 41).

Plasmid pRE8424 contains *rep*, a DSO, and an SSO, all of which are found universally on RCR plasmids; their nucleotide sequences are well conserved, especially in the pIJ101/pJV1 family. However, the organization of the DSO and SSO of pRE8424 is unique; the SSO of pRE8424 is located downstream of *rep* and the DSO is located downstream of the SSO (Fig. 2). The DSOs of RCR plasmids are usually located upstream of their *rep* genes or are sometimes embedded within the coding sequences of *rep*; most SSOs are located a short distance upstream of the DSOs (19).

Several types of SSOs, such as SSO_A, SSO_U, SSO_T, and SSO_W, have been identified based on their predicted secondary structures (19), and the SSO of pRE8424 likely belongs to SSO_A. Interestingly, rifampin, an inhibitor of cellular RNA polymerase, did not affect the conversion of ssDNA to dsDNA (Fig. 4A), suggesting that RNA polymerase might have no role or a limited role in the lagging-strand synthesis of pRE8424. In most RCR plasmids, cellular RNA polymerase plays an important role in lagging-strand synthesis (6), but only one plasmid, pWVO1, has been reported to have the synthesis of the lagging-strand carried out in an RNA polymerase-independent manner (32). IR I of pRE8424 seems to fulfill the conditions of an SSO, but it was not strongly involved in the replication of lagging-strand synthesis in *R. erythropolis* (Fig. 4A). IR I might function as an accessory SSO in other host cells to enhance the horizontal transmission of the plasmids or to stabilize the plasmids in the cell. We need to investigate further the lagging-strand synthesis mechanism of pRE8424 by using an in vitro replication system.

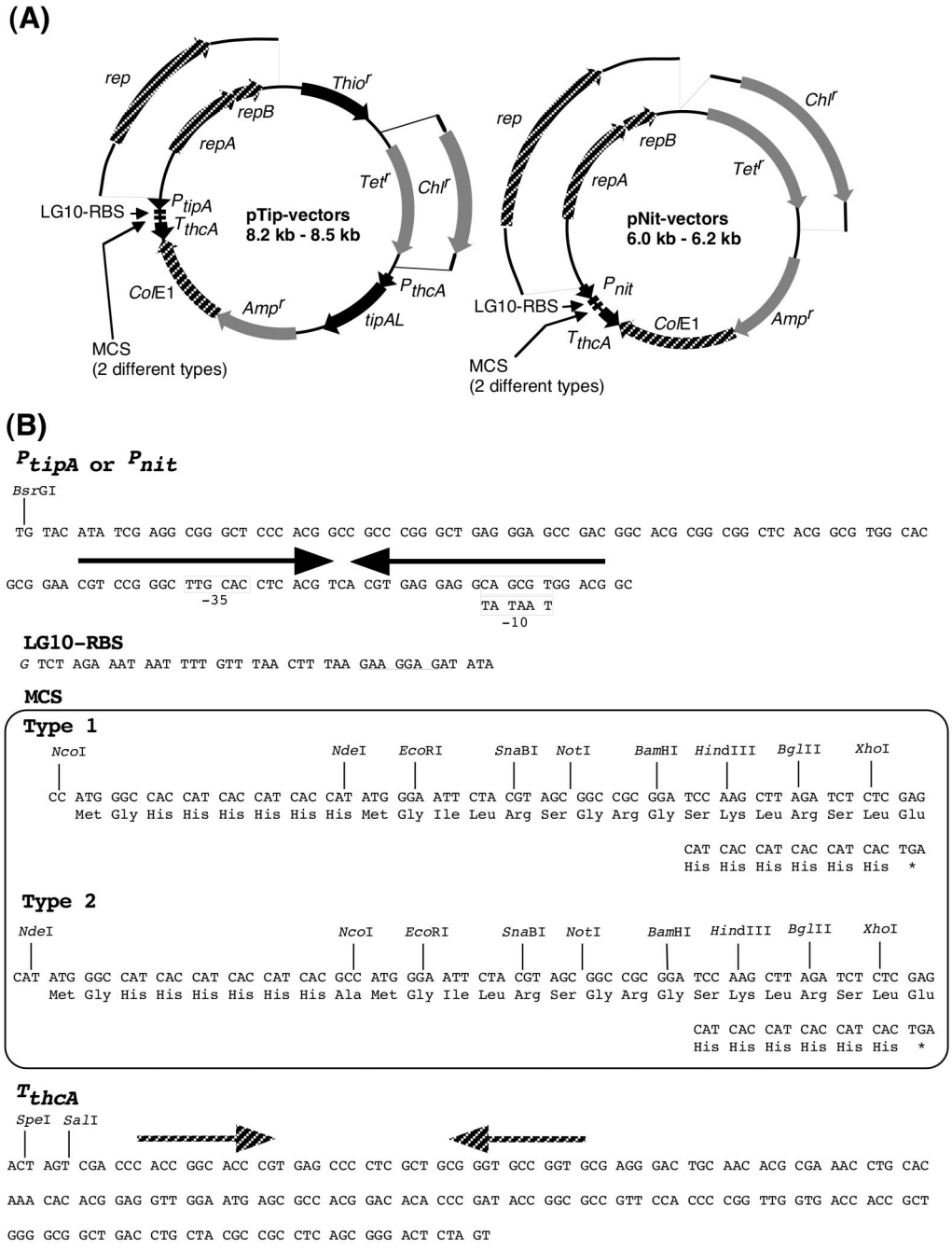


FIG. 5. Schematic maps and multiple cloning site sequences of pTip and pNit vectors. (A) Schematic maps of pTip and pNit vectors. *Thio*^{r}, thiostrepton resistance gene; *Tet*^{r}, tetracycline resistance gene; *Chl*^{r}, chloramphenicol resistance gene (each pTip and pNit vector has either *Tet*^{r} or *Chl*^{r}); P_{thcA}, *thcA* promoter that transcribes the *tipAL* gene constitutively; *Amp*^{r}, ampicillin resistance gene; *Col*E1, replication origin for *E. coli*; T_{thcA}, *thcA* transcriptional terminator; MCS, multiple cloning site (each pTip and pNit vector has either MCS type 1 or MCS type 2); P_{tipA}, *tipA* promoter; P_{nit}, *nit* promoter; LG10-RBS, RBS derived from gene 10 of a bacteriophage (10, 26); *repAB*, minimum region derived from pRE2895 for autonomous replication of the plasmid in *R. erythropolis*; *rep*, minimum region derived from pRE8424 for autonomous replication of the plasmid in *R. erythropolis* (each pTip and pNit vector has either *repAB* or *rep*). (B) Sequences of P_{tipA}, P_{nit}, LG10-RBS, multiple cloning site, and T_{thcA}. The imperfect IR sequence in the *tipA* promoter region is indicated by a solid arrows. The −35 and −10 hexamers are boxed. The Shine-Dalgarno sequence in the RBS is underlined. Transcription starts at the first "G" nucleotide (in italics) in the RBS. Hatched arrows indicate the positions of the perfect IR sequences in the *thcA* terminator. All of the restriction enzyme sites in the multiple cloning site, except for SnaBI (NcoI, NdeI, EcoRI, NotI, BamHI, HindIII, BglII, XhoI, SpeI, and SalI), are unique. Relationships between combinations of the genetic elements and the names of 16 expression vectors are indicated in Table 1.

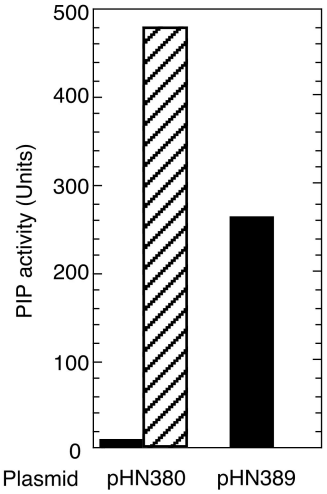


FIG. 6. Properties of P_{tip} and P_{nit}. *R. erythropolis* JCM3201 cells transformed with pHN380 or pHN389 were cultured, PIP was expressed, and the peptidase activity was monitored as described previously (26). Hatched bar, PIP activity from thiostrepton-treated cultures; solid bars, activity from untreated cultures.

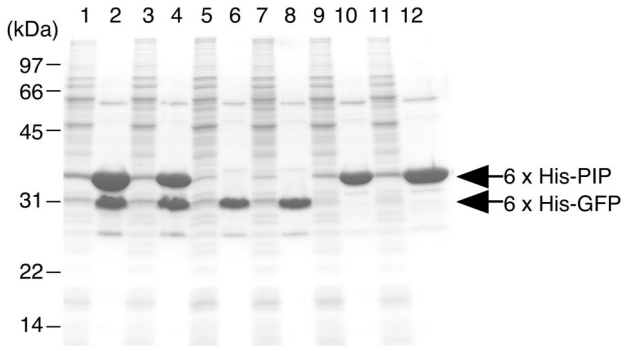


FIG. 7. Coexpression and purification of six-His–GFP and six-His–PIP. *R. erythropolis* JCM3201 was cotransformed with pHN425 and pHN389 (lanes 1 and 2) and with pHN426 and pHN409 (lanes 3 and 4). As controls, *R. erythropolis* JCM3201 was also transformed with either pHN425 (lanes 5 and 6), pHN426 (lanes 7 and 8), pHN389 (lanes 9 and 10), or pHN409 (lanes 11 and 12). Expressed GFP and/or PIP were purified by Ni-nitrilotriacetic acid superflow (see Materials and Methods). Each sample was prepared from 50 ml of culture medium. Cell extracts (odd-numbered lanes) and purified proteins (even-numbered lanes) were analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis followed by staining of the gel with Coomassie brilliant blue G-250.

Some plasmids originating from the pIJ101/pJV1 family are known to be broad-host-range plasmids (19), but pRE8424 derivatives such as pNit-RT2 or pNit-RC2 could not be maintained in *R. ruber* or *R. rhodochrous*. We wanted to exclude elimination by a restriction-modification system, which are widespread in *Streptomyces* spp. and which restrict the entry of DNA isolated from *E. coli* (24). The presence of a restriction-modification system in *R. rhodochrous* has also been suggested (12). Therefore, pNit-RT2 and pNit-RC2 plasmids isolated from either *R. erythropolis* JCM3201 or methylation-negative *E. coli* GM2163 (New England BioLabs, Inc.) were used for the transformation of *R. ruber* or *R. rhodochrous*, but again no transformants were obtained. It is likely that RCR plasmids require the host ssDNA binding protein, DNA ligase, and DNA gyrase for their replication (19). Those proteins in *R. ruber* or *R. rhodochrous* might not function with pRE8424. Transformation efficiencies might be correlated to the closeness of the species because a 16S rRNA analysis of *Rhodococcus* spp. has revealed that *R. rhodochrous* and *R. ruber* are classified into a group (group I) that is distant from the group of *R. erythropolis* and *R. opacus* (group IV), and *R. fascians* (group III) is more closely related to group IV (30).

In the future, we should investigate the structural stability and the behavior of the transformed plasmids in *Rhodococcus* for three reasons. First, two plasmids originating from pRE2895 were incompatible in *R. erythropolis*, but cotransformants appeared frequently (Table 3). Ikeda and Katsumata (15) have reported a similar result for *Corynebacterium glutamicum*; when two incompatible plasmids, one of which also carried a DNA fragment originating from the host cell, were introduced under selection with two corresponding antibiotics, cotransformants frequently appeared. In those transformants, the plasmid carrying the DNA fragment of the host cell was integrated into the genome via homologous recombination. Second, even when compatible plasmids were used for transformation, a slight reduction in transformation efficiency (about two to three times) was observed. This might be due to the procedure for the preparation of competent cells for electroporation; when competent cells were prepared from cells containing resident plasmids, chloramphenicol was always added to the culture medium to maintain the resident plasmids. Nevertheless, we cannot exclude the possibility of incompatibility or the joint instability of plasmids containing different replication origins. Third, RCR plasmids are historically used to construct cloning vectors for *Bacillus* spp. and *Staphylococcus* spp. but are rather unstable in these cells (11). When

TABLE 3. Cotransformation efficiencies for *R. erythropolis* JCM3201

Resident plasmid	Incoming plasmid	CFU/μg of DNA on tetracycline plate	% Chloramphenicol-resistant colonies[a] (n = 200)	% Tetracycline- and chloramphenicol-resistant colonies[a] (n = 200)
None	pNit-QT2	3.2×10^5		
pNit-QC2	pNit-QT2	2.0×10^3	35	29
pNit-RC2	pNit-QT2	1.3×10^5	100	100
None	pNit-RT2	4.4×10^4		
pNit-QC2	pNit-RT2	3.3×10^4	100	100
pNit-RC2	pNit-RT2	2.4×10^2	56	0

[a] Percentage of resistant colonies after second transformation.

heterologous DNA fragments are fused to RCR plasmids, they sometimes experience unexpected deletion or a reduced stability. This is due to the recombinogenic property of ssDNA intermediates, and in addition, θ-type replication generally results in more plasmid stability than the rolling circle mode of replication (11). We have not determined the integrity and stability of pRE8424 derivatives in *Rhodococcus*.

In this paper, we described eight inducible expression vectors (pTip vectors) and eight constitutive expression vectors (pNit vectors) for expressing recombinant proteins in several *Rhodococcus* species. The most important features of these vectors are their capabilities of cotransformation and of the coexpression of proteins in a single cell. These features can be used to characterize protein complexes comprising multiple components and enzymes involved in consecutive reactions in metabolic pathways. Protein coexpression can be achieved with one expression vector by cloning multiple genes of interest into one plasmid, but construction procedures for expression vectors are complicated because of the problem of restriction enzyme sites, of multiple RBS cloning, or of multiple promoter cloning.

The mutation in the −10 region of P_{tipA} resulted in the loss of thiostrepton-controlled transcription (Fig. 6). Because this region is highly secondarily structured and is the binding site for the TipAL protein (14), the destruction of the ordered structure may convert the inducible promoter, P_{tipA}, into the constitutive promoter, P_{nit}.

In *Rhodococcus*, some expression vectors using the *rrn* promoter and the *dsz* promoter have been constructed (3, 25), but the expression levels of proteins from these expression vectors remain unclear. The expression levels of proteins from pNit vectors were comparable to those from pTip vectors (Fig. 6) (26), and pNit vectors can express proteins even at 4°C (data not shown) just as pTip vectors do (26). We found that about several milligrams of recombinant proteins can be purified from pTip and pNit vector expression (Fig. 7) (26). Our expression vectors can be used to overexpress industrially important enzymes derived from specific *Rhodococcus* strains or to express enzymes to improve the metabolic pathway of a specific *Rhodococcus* strain.

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