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Isolation of two plasmids, pRET1100 and pRET1200, from *Rhodococcus erythropolis* IAM1400 and construction of a *Rhodococcus–Escherichia coli* shuttle vector

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With the aim of being able to co-express multiple genes, I searched for novel compatible plasmids and isolated two plasmid species, pRET1100 and pRET1200, from *Rhodococcus erythropolis* IAM1400. Sequencing analysis revealed that the pRET1100 plasmid is a double-stranded DNA molecule of 5444 bp with two possible open reading frames (ORFs), *repT* and *div*, and three minor ORFs. The cryptic replication protein, RepT, is not highly homologous to those from other plasmids that have been reported. The *Rhodococcus–Escherichia coli* shuttle vector pRET1102 was transformed into *R. erythropolis* JCM2895 harboring the pRE2895 plasmid. The recombinant *R. erythropolis* JCM2895 harbored two plasmid species. These results suggest that plasmid derivatives of pRET1100 and pRE2895 are fully compatible in *R. erythropolis*. I determined the minimum region of pRET1100 required for autonomous replication in *R. erythropolis* and constructed a high-copy plasmid, pRET1129, in *R. erythropolis*.

[**Key words:** Shuttle vector; *Rhodococcus*; Plasmid; Compatibility; pRET1100; High copy]

Many *Rhodococcus* strains contain diverse enzymes that are beneficial for manufacturing industries. In particular, *Rhodococcus erythropolis* tolerates organic solvents (1) and is utilized in the industrial production of chiral building blocks, pharmaceuticals (2,3), and chemicals (4). However, genetic tools are needed to further analyze and utilize *Rhodococcus* in manufacturing industries.

Plasmids capable of replicating in *R. erythropolis*, including pRC4 (5), pAL5000 (6), pRE2895 (7), and pRE8424 (8), have been reported by some researchers. In addition, using plasmids isolated from Actinomycetes, *R. erythropolis–Escherichia coli* shuttle vectors have been constructed. However, there have been few studies concerned with identifying a compatible expression vector other than pRE8424 (8).

Compatible expression vectors are advantageous in that they can be used to produce compounds from bacteria that co-express multiple genes (5). Therefore, I sought to discover novel compatible plasmids for co-expressing multiple genes and isolated two plasmid species, pRET1100 and pRET1200, from *R. erythropolis* IAM1400. This paper describes the new cryptic pRET1100 plasmid and a shuttle vector that is compatible with pRE2895.

MATERIALS AND METHODS

Strains and plasmids The properties of the strains and plasmids used in this study are summarized in Tables 1 and 2. *R. erythropolis* strains IAM1400 and IAM1503 were obtained from the Institute of Applied Microbiology (Tokyo, Japan). *R. erythropolis* strains JCM2893, JCM2894, and JCM2895 were obtained from the Japan Collection of Microorganisms (Ibaraki, Japan). *E. coli* and *R. erythropolis* were cultured in Luria-Bertani broth (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl) in the presence or absence of appropriate antibiotics. The antibiotics used to select transformants in the culture media were 100 μg/ml ampicillin, 100 μg/ml kanamycin, and 30 μg/ml chloramphenicol. The preparation of plasmids from *R. erythropolis* followed the method of Denis-Larose et al. (9).

Enzymes and chemicals All restriction enzymes and DNA modification enzymes were purchased from Toyobo Co., Ltd. (Osaka, Japan), New England Biolabs, Inc. (Ipswich, MA, USA), and Roche Molecular Systems, Inc. (Basel, Switzerland). All chemicals were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), Sigma-Aldrich (St. Louis, MO, USA), and Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Standard genetic manipulations Cloning was performed by standard genetic manipulation (10). The transformation of *R. erythropolis* followed the method of Hirasawa et al. (11). The transformation of *E. coli* was performed with the *E. coli* Transformation Buffer Set (Zymo Research Corp., Irvine, CA, USA). Genomic DNA was prepared with a Genomic DNA Buffer set and Genomic-tip 500/G (Qiagen, Hilden, Germany). PCR fragments were prepared with KOD-plus- (Toyobo Co., Ltd.).

Sequence determination and analysis pRET1100 and pRET1200 DNAs were digested with the appropriate restriction enzymes and then inserted into the *E. coli* vector pBluescript II KS (−). Determination of the DNA sequences of the plasmid inserts was accomplished by the primer walking method with an ABI PRISM 310NT DNA Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). Sequence assembly and analysis were performed with Genetyx (Genetyx Corp., Tokyo, Japan) and DNASIS Pro (Hitachi Software Corp., Tokyo, Japan).

Construction of the *Rhodococcus–E. coli* shuttle vectors The construction scheme for the shuttle vectors is shown in Fig. 1. To construct the pRET1102 plasmid, the DNA of pRET1100 was digested with *Alw*44 I, blunt-ended, and ligated with pHSG299 digested with *Hinc* II. To construct the pRET1200 plasmid, the DNA of pRET1200 was digested with *Bsp*LU11 I, blunt-ended, and ligated with pHSG299 digested with *Hinc*II.

Estimation of plasmid copy number Estimation of plasmid copy number was performed using the methods of Projan et al. (12) and Nakashima and Tamura (8). To estimate DNA band intensities, ImageJ was used (National Institutes of Health, Bethesda, MD, USA).

Nucleotide sequence accession numbers The sequence data for the pRET1100 and pRET1200 plasmids have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers LC331663 (pRET1100) and LC331662 (pRET1200).

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TABLE 1. Bacterial strains used in this study.

Species	Strain	Source	Application
E. coli	JM109	Toyobo Co., Ltd.	General cloning
	DH5alpha	Toyobo Co., Ltd.	General cloning
R. erythropolis	IAM1400	Institute of Applied Microbiology	Source of pRET1100 and pRET1200
	IAM1503	Institute of Applied Microbiology	Source of pRET1300 and pRET1400
	JCM2893	Japan Collection of Microorganisms	Source of pRET1500 and pRET1600
	JCM2894	Japan Collection of Microorganisms	Source of pRET1700 and pRET1800
	JCM2895	Japan Collection of Microorganisms	Source of pRET0500, host strain to transform with the shuttle vector
	MAK-34	This study	Host strain to transform with the shuttle vector

RESULTS

Isolation of plasmids from *R. erythropolis* I isolated cryptic circular plasmids from *R. erythropolis* strains IAM1400, IAM1503, JCM2893, and JCM2894. *R. erythropolis* IAM1400 harbored two plasmid species, designated as pRET1100 and pRET1200. These plasmids were the nearly same size (5.4 kb). pRET1100 harbors one recognition site for *Kpn*I and no recognition site for *Pst*I, while pRET1200 harbors one recognition site for *Pst*I and no recognition site for *Kpn*I. After digestion of these plasmids with *Pst*I or *Kpn*I, the resulting fragments were separated by agarose gel electrophoresis (Fig. 2).

When the pRET1200 DNA was digested with *Pst*I, the resulting amount of pRET1200 linear DNA was the same as that of the remaining pRET1100 closed circular DNA (Fig. 2, lane 2) and was approximately half the amount of the undigested pRET1100 and pRET1200 closed circular DNA (Fig. 2, lane 1). After *Pst*I treatment, the pRET1200 linear DNA (Fig. 2, lane 2) was able to be digested with ATP-dependent DNase, which selectively hydrolyzes linear dsDNA to deoxynucleotides, but the remaining pRET1100 DNA was not digested (data not shown). In contrast, the *Kpn*I-digested pRET1100 linear DNA (Fig. 2, lane 3) was able to be digested with ATP-dependent DNase, but the remaining pRET1200 DNA was not (data not shown). These results suggest that both pRET1100 and pRET1200 are circular closed dsDNAs in *R. erythropolis*.

R. erythropolis strains IAM1503, JCM2893, and JCM2894 each harbored two plasmid species, designated as pRET1300 and pRET1400, pRET1500 and pRET1600, and pRET1700 and pRET1800, respectively. The size of each plasmid was 5.4 kb (Table 2). I also isolated pRE2895 (7) from *R. erythropolis* JCM2895, which harbored one plasmid species.

The pRET1100 and pRET1200 DNAs were digested with a variety of restriction enzymes (Table 3), as were the pRET0500 (pRE2895), pRET1300, pRET1400, pRET1500, pRET1600, pRET1700, and

TABLE 2. Plasmids used in this study.

Plasmid	Characteristics	Source/Reference
Cloning vectors		
pUC18	Ap^{r}[a]	Takara Bio Inc.
pHSG299	Km^{r}[b]	Takara Bio Inc.
pHSG398	Cm^{r}[c]	Takara Bio Inc.
pBluescript II KS(−)	Ap^{r}	Toyobo Co., Ltd.
pRET vector		
pRET0500	Same as pRE2895	(7)
pRET1100	5.4 kb plasmid isolated from *R. erythropolis* IAM1400	This study
pRET1200	5.4 kb plasmid isolated from *R. erythropolis* IAM1400	This study
pRET1300	5.4 kb plasmid isolated from *R. erythropolis* IAM1503	This study
pRET1400	5.4 kb plasmid isolated from *R. erythropolis* IAM1503	This study
pRET1500	5.4 kb plasmid isolated from *R. erythropolis* JCM2893	This study
pRET1600	5.4 kb plasmid isolated from *R. erythropolis* JCM2893	This study
pRET1700	5.4 kb plasmid isolated from *R. erythropolis* JCM2894	This study
pRET1800	5.4 kb plasmid isolated from *R. erythropolis* JCM2894	This study
pRET1101	Ap^{r}; DNA fragment of pRET1100 digested with *Alw*44 I and blunt ended in *Sma* I site of pUC18	This study
pRET1102	Km^{r}; DNA fragment of pRET1100 digested with *Alw*44 I and blunt ended in *Hinc* II site of pHSG299	This study
pRET1103	Cm^{r}; DNA fragment of pRET1100 digested with *Alw*44 I and blunt ended in *Hinc* II site of pHSG398	This study
pRET1201	Ap^{r}; DNA fragment of pRET1200 digested with *Bsp*LU11 I and blunt ended in *Sma* I site of pUC18	This study
pRET1202	Km^{r}; DNA fragment of pRET1200 digested with *Bsp*LU11 I and blunt ended in *Hinc* II site of pHSG299	This study
pRET1203	Cm^{r}; DNA fragment of pRET1200 digested with *Bsp*LU11 I and blunt ended in *Hinc* II site of pHSG398	This study
pRET1123	Km^{r}; 2.7 kb DNA fragment of pRET1102 digested with *Bam*H I and *Hinc* II in *Bam*H I and *Hinc* II sites of pHSG299	This study
pRET1127	Km^{r}; PCR fragment of pRET1100 (nucleotides 468 to 2768) in *Hinc* II site of pHSG299	This study
pRET1128	Km^{r}; PCR fragment of pRET1100 (nucleotides 468 to 2684) in *Hinc* II site of pHSG299	This study
pRET1129	Km^{r}; PCR fragment of pRET1100 (nucleotides 628 to 3144) in *Hinc* II site of pHSG299	This study
pRET1130	Km^{r}; PCR fragment of pRET1100 (nucleotides 877 to 3144) in *Hinc* II site of pHSG299	This study

[a] Ap^{r}, ampicillin resistance.
[b] Km^{r}, kanamycin resistance.
[c] Cm^{r}, chloramphenicol resistance.

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