Note

Construction of a New Shuttle Vector for *Zymomonas mobilis*

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Zymomonas mobilis is a facultative anaerobic gram-negative bacterium, which has considerable potential for industrial alcohol fermentation as shown by Rogers *et al.*[1] However, it has the disadvantage that the range of fermentable sugars is restricted to glucose, fructose and sucrose.[2] A cloning vector for *Z. mobilis* is needed to extend the substrate range of *Z. mobilis* by genetic manipulation. Workers at several laboratories have shown that broad host range plasmids for gram-negative bacteria can be conjugally transferred into *Z. mobilis*.[3~5] Browne *et al.* have reported that *Z. mobilis* is transformed by a 28-kilobase pair (kb) cointegrate plasmid between an *Escherichia coli* plasmid and a cryptic plasmid of *Z. mobilis* ATCC 29191.[6] However, these plasmids are not suitable vectors for the introduction of foreign genes into *Z. mobilis*, since they have large molecular weights.

Tonomura *et al.* have shown that *Z. mobilis* ATCC10988 contains three kinds of small plasmids in size, 1.7, 2.7 and 3.9 kb.[7] Misawa *et al.* have reported that the 1.7 kb plasmid can be subdivided into three species.[8] Okita *et al.* have reported the complete DNA sequence of one of the 1.7 kb plasmids, pZM81A.[9] A recent study by Tonomura *et al.* has shown that hybrids between the 3.9 kb plasmid (designated as pZM3) of *Z. mobilis* ATCC10988 and *E. coli* vector pACYC184 can be used as shuttle vectors in *E. coli* and *Z. mobilis*.[10] These hybrid plasmids possess only a single selectable antibiotic marker gene and only a few unique restriction enzyme sites, although they have small molecular weights (7.9 kb).

We report here the construction of a useful shuttle vector, pZA22, which replicates in both *E. coli* and *Z. mobilis*, with two antibiotic marker genes. pZA22 was constructed by joining *Z. mobilis* ATCC10988 2.7 kb plasmid (designated as pZM2,[11] corresponding to plasmid pRUT883 of Stokes *et al.*[12]) to pACYC184, and showed 2 ~ 3 times as high a copy number as that of the shuttle vectors of Tonomura *et al.*,[10] in *Z. mobilis*.

E. coli was grown in L-broth medium composed of 1% tryptone, 0.5% yeast extract and 1% NaCl, and *Z. mobilis* in RM medium[3] composed of 1% yeast extract (Oxoid), 2% glucose and 0.2% KH_2PO_2. Plasmid DNA was isolated from *E. coli* or *Z. mobilis* by the procedure described by Birnboim and Dóly,[13] and purified by phenol/chloroform extraction and ethanol precipitation. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim, New England Bio-Labs, or Takara Shuzo Co., Ltd., and reactions were carried out under the conditions recommended by the suppliers. Digested DNAs were analysed by agarose gel electrophoresis.

Restriction enzyme analysis was carried out on plasmid pZM2 isolated from *Z. mobilis* ATCC10988. The restriction map is shown in Fig. 1A. pZM2 had no sites for *Acc*I, *Aha*II, *Bam*HI, *Ban*II, *Bcl*I, *Bgl*II, *Bst*EII, *Cla*I, *Eco*52I, *Eco*RI, *Eco*RV, *Hae*II, *Hin*cII, *Hgi*AI, *Hpa*I, *Kpn*I, *Mfl*I, *Mlu*I, *Nco*I, *Nhe*I, *Nsi*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sac*II, *Sal*I, *Sma*I, *Sna*BI, *Spe*I, *Stu*I, *Xba*I or *Xho*I. Digestion of pZM2 or pACYC184 with *Ava*I pro-

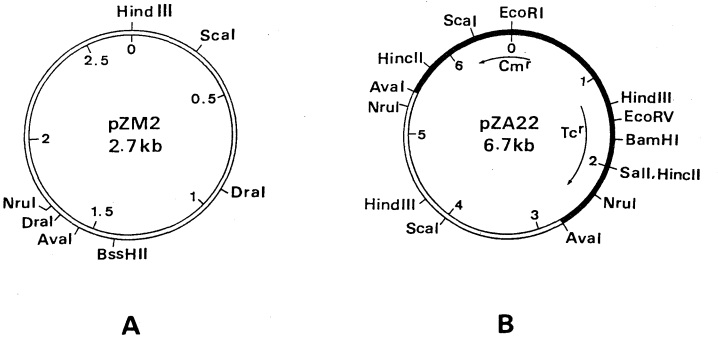

FIG. 1. Restriction Maps of pZM2 (A) and pZA22 (B).

pZA22 was constructed by inserting the *Ava*I fragment of pZM2 into the *Ava*I site of pACYC184. Symbols: □, pZM2 DNA; ■, pACYC184 DNA. The numbers indicate kilobase pairs (kb)

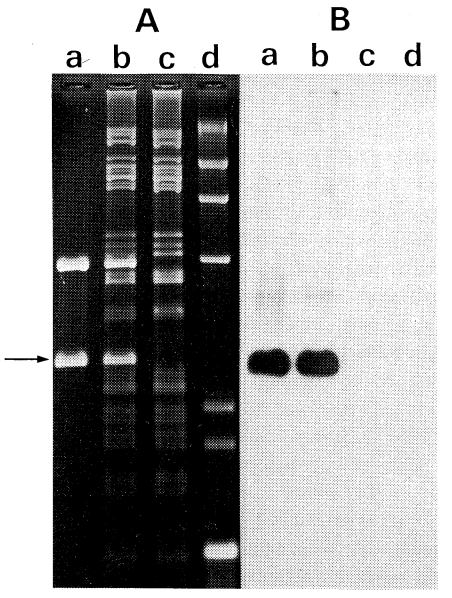

FIG. 2. (A) Agarose Gel Electrophoresis of *Ava*I-digested Plasmid DNAs in a 1.0% Agarose Gel.

*Ava*I digests of, a, plasmid pZA22 DNA; b, plasmid DNAs prepared from *Z. mobilis* NRRL B-14023 transconjugant of pZA22; c, plasmid DNAs prepared from *Z. mobilis* NRRL B-14023; d, molecular weight standards (λ-*Hin*dIII/$\phi \times 174$-*Hae*III): 23.1 kb, 9.4 kb, 6.6 kb, 4.4 kb, 2.3 kb, 2.0 kb and 1.4 kb (top to bottom). Arrow indicates the *Ava*I fragment of pZM2.

(B) Southern Hybridization of (A) Using Plasmid pZM2 *Ava*I Fragment as a Probe.

duced a single fragment. 1 μg of *Ava*I-cut pZM2 DNA was ligated with 0.2 μg of *Ava*I-cut pACYC184 DNA with T4 DNA ligase, and *E. coli* C600 was transformed. After the restriction enzyme analysis of the plasmids isolated from these transformants, we obtained the desired hybrid plasmid, in which the *Ava*I fragment of pZM2 was inserted into the *Ava*I site of pACYC184, designated as pZA22 (Fig. 1B).

To examine whether pZA22 can be introduced into *Z. mobilis* NRRL B-14023 or not, conjugal transfer with helper plasmid pRK2013 was carried out, as described by Tonomura *et al.*[10] the transconjugants of *Z. mobilis* NRRL B-14023 harboring pZA22 were obtained on a RM agar plate containing chloramphenicol (Cm) 100 μg/ml and/or tetracycline (Tc) 15 μg/ml, and nalidixic acid 100 μg/ml. The transfer frequency of pZA22 was 7.6×10^{-7}/donor or 1.0×10^{-6}/recipient. Plasmids isolated from these *Zymomonas* transconjugants were subjected to restriction enzyme analysis and Southern hybridization analysis[14] using the ^{32}P-labeled pZM2 *Ava*I fragment as a probe. The result showed that pZA22 was maintained stably in the transconjugants, in addition to the endogeneous plasmids contained in *Z. mobilis* NRRL B-14023 (Fig. 2). There was no indication of deletion or segregation of pZA22 in the transconjugants.

pZM2 is cleaved with a single site by *Hin*dIII or *Sca*I digestion as shown in Fig. 1A. The fragments digested with *Hin*dIII and *Sca*I were ligated with pACYC184 digested with *Hin*dIII and *Sca*I, respectively. These hybrid plasmids were obtained after transformation in *E. coli* C600 and the restriction enzyme analysis of plasmids isolated from the transformants. These hybrid plasmids were not introduced into *Z. mibilis* by the conjugal transfer with pRK2013. Therefore, it was suggested that there might be *Hin*dIII and *Sca*I sites within the region which was necessary for replication in *Zymomonas*.

The ratio of the copy number of pZA22 to the vector of Tonomura *et al.*[10] (a hybrid plasmid of pZM3 and pACYC184, designated as pZA32) in *Zymomonas* was analysed by a modification of the method of projan *et al.*[15] as follows: Plasmid DNAs were prepared from *Z. mobilis* NRRL B-14023 transconjugant harboring pZA22 and that harboring pZA32, which were the same with regard to cell number and growth phase, digested by restriction enzymes, and electrophoresed in a 1.0% agarose gel. Photographs of the gels were taken under irradiation by ultra-violet light, and negative films were analysed by a densitometer (Shimadzu CS-930). As a result, pZA22 showed 2 ~ 3 times as high a copy number as that of pZA32 in *Z. mobilis* NRRL B-14023.

pZA22 has a small molecular weight (6.7 kb), and a relatively high copy number. pZA22 has both chloramphenicol and tetracycline resistance marker genes, and contains unique restriction sites, for *Eco*RI, *Eco*RV, *Bam*HI and *Sal*I within the drug-resistance marker genes. If a foreign gene is inserted into these sites with appropriate orientation, the expression of the gene may result from transcriptional read-through from the promoters of the marker genes. Therefore, pZA22 should be useful as a vector which can clone foreign genes in *Z. mobilis*.

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