Short Communication

Shuttle Vectors for Zymomonas mobilis

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Zymomonas mobilis is a facultative anaerobic gram-negative bacterium, the biology of which has been reviewed by Swings et al. It almost produces the theoretical yield of ethanol from glucose, and exhibits higher ethanol productivity than industrial yeasts. It is assumed, therefore, to be potentially useful for the commercial production of ethanol. However, the range of substrates utilized by the organism is restricted to glucose, fructose and sucrose, and this severely limits its commercial use. To increase the substrate range of Z. mobilis by recombinant DNA techniques, it is necessary to construct a vector for Z. mobilis.

Recent studies have shown that plasmids having a broad-host range can be conjugally transferred into Z. mobilis. Skotnicki et al. reported that RP4 and R68.45 could be transferred from E. coli into Z. mobilis. Carey et al. and Goodman et al. reported that RP1, containing a lactose transposon, Tn951, was transferred into Z. mobilis and stably maintained in it. Browne et al. have reported that pNSW601 (a 28-kilobase (kb) cointegrate plasmid formed in vivo in Z. mobilis between an E. coli plasmid and a cryptic plasmid of Z. mobilis) transformed Z. mobilis and was stably maintained in it. However, these are not suitable vectors for the introduction of foreign genes into Z. mobilis, since they have large molecular weights. We have previously shown that Z. mobilis ATCC10988 contains three plasmids species of small molecular size, 1.7, 2.5 and 3.9 kb. Stokes et al. have confirmed the presence of both 1.7- and 2.5-kb plasmids but not the 3.9-kb plasmid. Misawa et al. have indicated that the 1.7-kb plasmid can be subdivided into three species.

In this paper, we report that shuttle vectors between Z. mobilis and E. coli were constructed by inserting the 3.9-kb plasmid (designated as pZM3) of Z. mobilis ATCC10988 into E. coli plasmid pACYC184, and that they were conjugally transferred into Z. mobilis from E. coli through a helper plasmid, pRK2013, and stably maintained in the host.

E. coli was grown in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% glucose, pH 7.2), and Z. mobilis in RM medium (2% glucose, 1% yeast extract, 0.2% KH₂PO₄, pH 6.0). Plasmid DNA was isolated from E. coli or Z. mobilis by the alkaline-SDS-method of Birnboim and Doly, and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation. DNA samples were subjected to electrophoresis in a standard vertical slab gel apparatus by the method of Meyers et al.

Plasmid pZM3 was digested with a variety of restriction endonucleases, and a physical map was made for pZM3 (Fig. 1a). It was consistent with the map proposed by Abe et al. Skotnicki et al. reported that RP4 and R68.45 could be transferred from E. coli into Z. mobilis. Carey et al. and Goodman et al. reported that RP1, containing a lactose transposon, Tn951, was transferred into Z. mobilis and stably maintained in it. Browne et al. have reported that pNSW601 (a 28-kilobase (kb) cointegrate plasmid formed in vivo in Z. mobilis between an E. coli plasmid and a cryptic plasmid of Z. mobilis) transformed Z. mobilis and was stably maintained in it. However, these are not suitable vectors for the introduction of foreign genes into Z. mobilis, since they have large molecular weights. We have previously shown that Z. mobilis ATCC10988 contains three plasmids species of small molecular size, 1.7, 2.5 and 3.9 kb. Stokes et al. have confirmed the presence of both 1.7- and 2.5-kb plasmids but not the 3.9-kb plasmid. Misawa et al. have indicated that the 1.7-kb plasmid can be subdivided into three species.

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Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. and used according to the recommendations of the supplier.

Plasmid pZM3 was digested with a variety of restriction endonucleases, and a physical map was made for pZM3 (Fig. 1a). It was consistent with the map proposed by Abe et al. To construct a hybrid plasmid, pZM3 and pACYC184 were separately digested with EcoRI, and then ligated with T4 ligase. E. coli C600 was transformed with the hybrid, and the hybrid DNA was examined with a variety of restriction endonucleases. The hybrids with both orientations of pZM3 inserted into pACYC184 were obtained, of which one is shown in Fig. 1b, which was designated as pZA31. The plasmid was 7.9-kb in size and showed resistance to tetracycline (Tc). By an analogous method, hybrid plasmids of pZA32 and pZA33 were prepared; pZA32 was constructed by joining pZM3 to pACYC184 at a
Fig. 1. Physical Maps of pZM3 (a), pZA31 (b), pZA32 (c) and pZA33 (d).

HindIII site, and pZA33 by joining pZM3 at a BglII site and pACYC184 at a BamHI site. Both plasmids showed resistance to chloramphenicol (Cm) and were 7.9 kb in size. Figures 1c and 1d show the physical maps of pZA32 and pZA33, respectively.

To transfer a hybrid from E. coli into Z. mobilis, the kanamycin (Km)-resistant helper plasmid, pRK2013, was used. Plasmid pRK2013 was conjugally transferred into E. coli HB101 (pro, leu, thi, lac y, Sm', end1, rec', r', m') carrying pZA31 by a membrane filter method,\(^{15}\) and a clone carrying both plasmids was obtained by selection with Km' and Tc'. The plasmids were obtained by agarose gel electrophoresis, as shown in Fig. 2, lane 3. Plasmid pZA31 was conjugally transferred from the clone of E. coli to Z. mobilis CP4 (NRRL B-14023) with resistance to nalidixic acid (Nal) by a membrane filter method; the filter was placed on a RM agar plate for 4 hr at 37°C and then the Zymomonas transconjugant was selected in the presence of Tc (30 μg/ml) and Nal (100 μg/ml). The plasmid was observed in transconjugants with endogenous plasmids of the CP4 strain, as shown in Fig. 2, lane 5. The transfer frequency of pZA31 was \(7.7 \times 10^{-6}/\text{donor}\). Plasmid pZA31 was also transferred into Z. mobilis IFO13756.
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Fig. 3. Reisolation of Shuttle Vectors.

Plasmid DNA was extracted, digested and then subjected to agarose gel electrophoresis. Lanes: (1) pZA31 DNA isolated from E. coli HB101; (2) pZA31 from Z. mobilis CP4; (3) pZA32 from E. coli HB101; (4) pZA32 from Z. mobilis CP4; (5) pZA33 from E. coli HB101; (6) pZA33 from Z. mobilis CP4. Each of the plasmids used in (1), (3) and (5) was transferred from Z. mobilis CP4 to E. coli HB101. Lanes (1) and (2) are EcoRI digests, (3) and (4) HindIII digests, and (5) and (6) PvuII digests. A 0.45-kb PvuII fragment of pZA33 is not visible in lanes (5) and (6).

at a frequency of $4.2 \times 10^{-7}$/donor by the same method. For the transfer of pZA32 and pZA33 to Z. mobilis CP4, transconjugants were selected in the presence of Cm (100 μg/ml) and Nal (100 μg/ml). Plasmid pZA32 and pZA33 were transferred into Z. mobilis CP4 at frequencies of $3.1 \times 10^{-6}$/donor and $2.5 \times 10^{-7}$/donor, respectively.

To determine the stabilities of pZA31, pZA32 and pZA33 in Z. mobilis CP4, the organism, after selection with Tc or Cm, was cultured for 13 hr in RM medium, and then the viable count was determined by plating the cells on RM plates. The proportion of plasmid-carrying cells was determined by replica-plating onto RM medium containing 30 μg/ml of Tc for pZA31 or 100 μg/ml of Cm for pZA32 and pZA33. The stabilities of pZA31, pZA32 and pZA33 determined by this method were 77, 56 and 38 percent, respectively.

The plasmids, pZA31, pZA32 and pZA33, could be transferred from Z. mobilis back to E. coli HB101 by transformation; the data for pZA31 are shown in Fig. 2, lane 6. To examine whether these plasmids are modified in Z. mobilis or not, the plasmids prepared from Z. mobilis CP4 and also from E. coli HB101 were digested with a restriction endonuclease, and then analyzed by agarose gel electrophoresis, as shown in Fig. 3. As the CP4 strain contained two species of an endogenous plasmid, many extra DNA bands were observed besides the DNA bands arising from the hybrid, as shown in Fig. 3, lanes 2, 4 and 6. The results were consistent with those expected from the cleavage map. This suggested that the plasmids were not modified in Z. mobilis.

The availability of shuttle vectors for use in E. coli and Z. mobilis will facilitate the genetic investigation of Z. mobilis and strain improvement of the organism by means of genetic engineering.

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