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Construction of Plasmid Vectors for *Zymomonas mobilis*

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*Zymomonas mobilis*의 Plasmid Vector 제조에 관한 연구

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In order to develop useful plasmid vectors for *Zymomonas* cells, attempts were made to isolate natural plasmids from *Z. mobilis* ATCC10988. Among a few plasmids isolated, a small plasmid of 3.9 Kb size was chosen and designated as pZM3. By introducing the replication origin of pZM3 into pBR325, a hybrid plasmid vector of 8.4 Kb size, pHZ22, was constructed. This vector contained chloramphenicol resistant gene as a selectable marker and proved to be conjugally transmissible and stably maintained in *Z. mobilis*. Tetracycline resistant gene was isolated from RP4 and introduced into pHZ22 to make a new vector called pHZT224 of 10.7 Kb size. Through a series of experiments, it was evident that these plasmid vectors containing selectable markers of chloramphenicol and tetracycline resistance were shuttle vectors functional in *Z. mobilis* as well as *E. coli*.

Zymomonas mobilis is a facultative anaerobic Gram-negative bacterium capable of producing large amounts of ethanol from glucose via Entner-Doudoroff pathway (1).

Compared with yeasts, this bacterium has been known to be more thermotolerant, ethanol-tolerant, osmotolerant and producing ethanol in three to five-fold higher rates (2,3). With these properties, *Z. mobilis* has been considered potentially useful for industrial production of ethanol.

Despite all these attractive characteristics of *Z. mobilis* cells for ethanol production, the range of substrate utilization by this microorganism is confined to only glucose, fructose and sucrose and does not include rather cheap industrial substances, viz. starch, cellulose and lactose (1). This fact imposes severe limitation of *Z. mobilis* on its commercial use. In this regard, it will be of particular importance to broaden the substrate range of *Z. mobilis* through genetic manipulation.

To achieve this goal, however, plasmid vectors for *Zymomonas* should be first constructed as cloning vehicles for genes coding for hydrolysing enzymes such as amylases and cellulases. Several investigations have been made under this strategy but only few, if any, vectors were proven suitable for *Z. mobilis* mostly due to their large molecular weights (4), lack of suitable selective markers (5), or *Zymomonas* replication origins (6,7).

In the present investigation, we have constructed a number of small size shuttle vectors containing replication origins of *Z. mobilis* and *E. coli* together

Key words: plasmid vectors, *Z. mobilis*, antibiotic markers, natural replicon, conjugation, shuttle vectors
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with suitable selective markers.

Materials and Methods

Bacterial strains and enzymes

Z. mobilis ATCC10988 and *Z. mobilis* ATCC31821 (ZM4) stock cultures were used. *E. coli* strains used were HB101 (hsdR, hsdM, recA13, supE44, lacZ4, leuB6, proA2, thi-1, rpsL) and C600 (thr-1, leu-6, thi-1, supE44, lacY1 and tonA21). Restriction endonuclease, DNA polymerase, Klenow fragment of DNA polymerase, T4 DNA ligase, T4 DNA kinase were from Bethesda Research Laboratories, Inc.

Media and reagents

E. coli was grown in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH7.2), and *Z. mobilis* in RM medium (2% glucose, 1% yeast extract, 0.2% KH_2PO_4, pH6.0) (8). All buffer solutions and reagents were prepared by following the methods described by Maniatis *et al.* (9).

Isolation of plasmid DNA

Plasmid DNAs were isolated from *E. coli* or *Z. mobilis* by the alkaline-SDS-method of Birnboim and Doly (10), 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.* (11).

Conjugation

Donor strains were first constructed by the supertransformation method (7, 8, 12). Donor and recipient strains were *E. coli* C600 (RP4, non-conjugative plasmid) and *Z. mobilis* ATCC31821. Donor and recipient cultures were grown separately to logarithmic phase in an unshaken culture in a standard medium, and mixed with donor to recipient ratio of 3:1. This mating mixture was centrifuged, resuspended in 50 m*l* RM broth, transferred to 0.45 μM Millipore filters on an RM agar plate, and incubated at 30 °C for 3hrs. This was resuspended by placing filters in a tube containing 0.5 m*l* of 0.85% saline and agitating the tube on a vortex. Resuspended cells were then spread on selective plates.

Transformation

Transformation of plasmid DNA into *E. coli* was performed by following the method described by Mandel and Higa (13) which was modified by Dagert and Ehrlich later (14). All steps of transformation were carried out at chilling temperature unless specified otherwise.

Southern blot hybridization

Plasmid DNA resolved on 0.7% agarose gels was transferred to a nitrocellulose filter by the Southern technique (15). The filter was hybridized with γ-(^{32}P)dATP-labelled probe prepared by kination (16).

Results

Isolation of natural plasmids from *Zymomonas*

A number of natural plasmid DNAs were isolated from strains of *Zymomonas* cells harvested in logarithmic phase. The patterns of plasmid DNAs and their sizes are shown in Fig. 1. It was found that *Z. mobilis* ATCC10988 harbored five different plasmids, whereas *Z. anaerobia* NCIB8277 two and *Z. mobilis* ATCC31821 only one. Among these na-

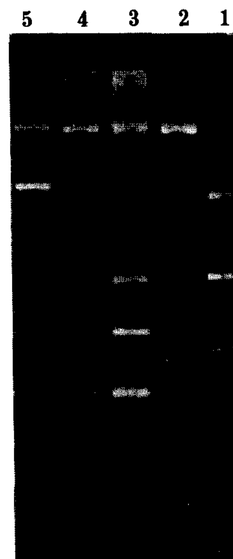

Fig. 1. Natural plasmids from various *Zmomonas* strains.
Lanes 1; pBR322, as a control, 2; plasmids from *Z. mobilis* ATCC31812, 3; plasmids from *Z. mobilis* ATCC10988, 4; plasmids from *Z. anaerobia* NCIB8227, 5; plasmids from *Z. mobilis* AG11

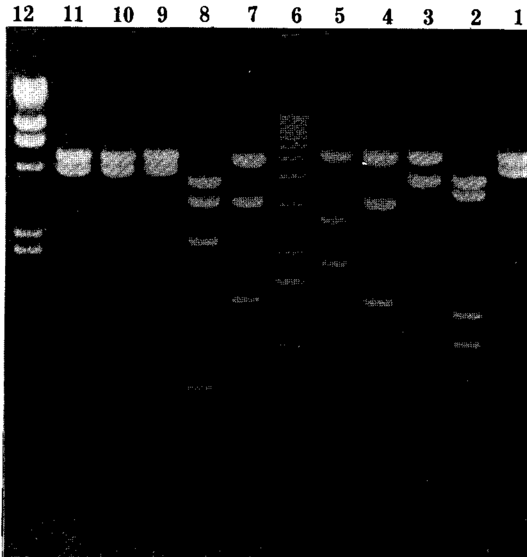

Fig. 2. 0.8% agarose gel electrophoresis patterns of hybrid plasmid pPZ2 after digestion with various restriction endonucleases.
Lanes 1; pPZ2 digested with AvaI, 2; AvaI+BamHI, 3; AvaI+BglII, 4; AvaI+ClaI, 5; AvaI+EcoRI, 6; 1 Kb ladder, the sizes of fragments, in Kb, from top to bottom are: 12.2, 11.2, 10.2, 9.2, 8.2, 7.1, 6.1, 5.0, 4.0, 3.0, 2.0, 1.6, 1.0 and 0.5., 7; AvaI+HineIII, 8; AvaI+PstI, 9; AvaI+PvuI, 10; AvaI+SmaI, 11; AvaI+XhoI, 12; λ cI857 S7 DNA digested with HindIII, the sizes of fragments, in Kb, from top to bottom are: 23.1, 9.42, 6.68, 4.36, 2.36, 2.03 and 0.56.

tural plasmids in various sizes, pZM3 from the strain ATCC10988 was selected for vector construction with respect to its suitable size of 3.9 Kb.

Physical map of pZM3

For the physical mapping of the plasmid pZM3, a hybrid plasmid was first constructed between pZM3 and pBR325. Both plasmids were cleaved at one site by PstI and ligated together. This new plasmid thus formed were designated as pPZ2. By digesting pPZ2 with several restriction enzymes (Fig. 2), the physical map of pZM3 was depicted. As shown in Fig. 3, it was found that pZM3 contained one cleavage site for AvaI, BamHI, BglII and PstI, and two for HindIII.

Antibiotic resistance of Zymomonas

Zymomonas strains were tested for antibiotic resistance in an attempt to introduce these resistant genes as useful selective markers. As shown in

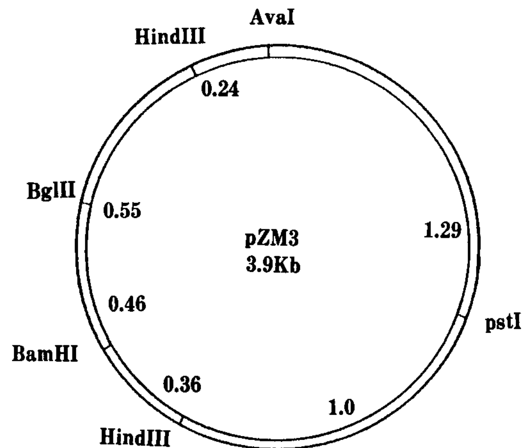

Fig. 3. Restriction map of pZM3.

Table 1, *Zymomonas* strains tested showed strong resistance against ampicillin (up to 300 μg/ml) and streptomycin (up to 1000 μg/ml) but sensitivity to chloramphenicol (10-25 μg/ml) and tetracycline (5 μg/ml).

Construction of plasmids pHZ22 and pHZT224

A trial was made to construct a hybrid plasmid between pZM3 and pBR325. Using HindIII as a restriction enzyme, two plasmids of different pZM3 fragment sizes (2.4 and 1.5 Kb) were constructed. Among these, the hybrid plasmid containing the large fragment was isolated and designated as

Table 1. Minimal inhibitory concentration (MIC)[a] of antibiotics to *Zymomonas* strains

Host strains	Antibiotics (μg/ml)			
	Ampicillin	Chloramphenicol	Tetracycline	Streptomycin
Z. mobilis ATCC10988	200	10	5	1000
Z. mobilis ATCC31812	300	25	5	1000
Z. anaerobia NCIB8227	100	20	5	1000

[a]: MIC is defined as the level of antibiotics (in μg/ml) which reduces survival of *Z. mobilis* to 50% of viable count on unsupplemented complete medium. Survival was determined by spotting 100 μl aliquots of overnight culture onto appropriately supplemented complete medium.

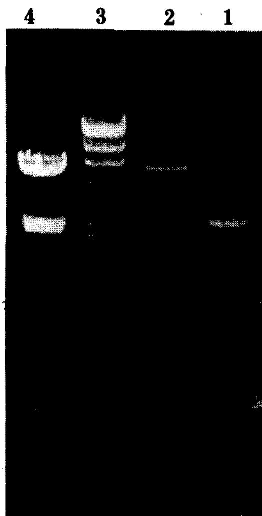

Fig. 4. Electrophoretic characterization of hybrid plasmid pHZ22.
Lanes 1; pZM3 digested with HindIII, 2; pBR325 digested with HindIII, 3; λ cI857 S7 DNA digested with HindIII, 4; pHZ22 digested with HindIII.

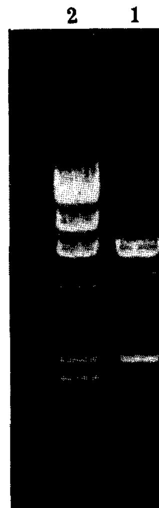

Fig. 5. Electrophoretic characterization of hybrid plasmid pHZT 224.
Lanes 1; pHZT224 digested with HindIII, 2; λ cI857 S7 DNA digested with HindIII.

pHZ22. The electrophoretic characterization of pHZ22 was demonstrated in Fig. 4. In order to introduce tetracycline resistant gene into pHZ22, another trial was made using a plasmid, RP4, as a source of tetracycline resistant gene. pHZ22 was digested with BamHI and partially digested with PvuII and a 6.7 Kb fragment was isolated. The tetracycline resistant gene (2.4 Kb) was also isolated from RP4 by double digestion with BglII and SmaI. A hybrid plasmid called pHZT224 was then formed by cohesive-blunt end ligation of these two fragments exploiting the property of enzyme compatibilities (BamHI and BglII, PvuII and SmaI). The electrophoretic characterization of pHZT224 thus formed was demonstrated in Fig. 5. In Fig. 6, the total constructing strategy of the plasmids pHZ22 and pHZT224 is illustrated.

Mobilization of pHZ22 and pHZT224 into host cells

Transfer of pHZ22 and pHZT224 into host cells was tested. In these experiments, RP4 was employed as a helper plasmid. The mobilization experiments were carried out by use of conjugation techniques. Streptomycin, chloramphenicol and tetracycline were used as selective markers. As shown

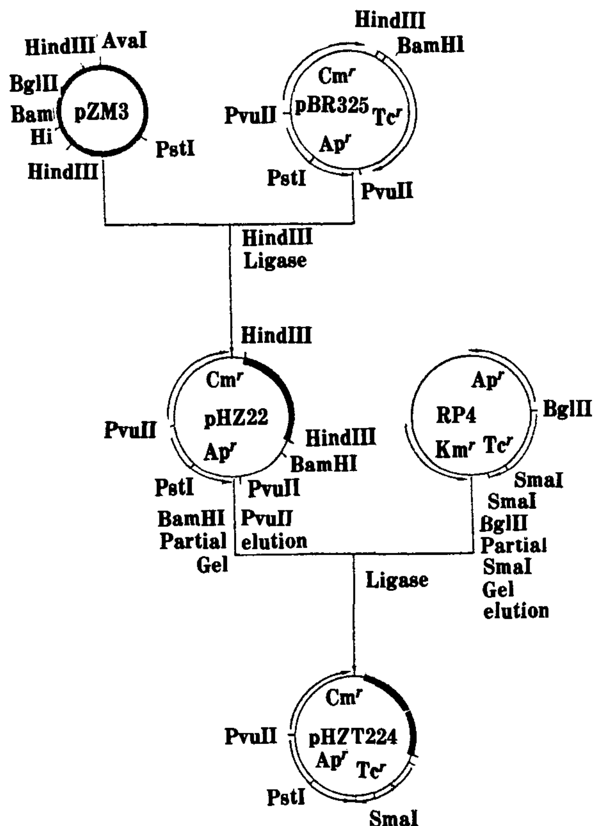

Fig. 6. Strategy for plasmid vector construction.

Table 2. Mobilization of pHZ22 and pHZT224 into host cells.

Donor cells (plasmids)	Recipient cells	Selective markers[a]	Mobilization
E. coli C600 (pBR325)	*E. coli* HB101	Sm, Cm	impossible
E. coli C600 (pBR325, RP4)	*Z. mobilis* ATCC31812	Sm, Cm	possible
E. coli C600 (pHZ22, RP4)	*E. coli* HB101	Sm, Cm	possible
E. coli C600 (pHZ22, RP4)	*Z. mobilis* ATCC31812	Sm, Cm	possible
E. coli C600 (pHZT224, RP4)	*E. coli* HB101	Sm, Cm, Tc	possible
E. coli C600 (pHZT224, RP4)	*Z. mobilis* ATCC31812	Sm, Cm, Tc	possible

[a]: Sm represents Streptomycin, Cm; Chloramphenicol and Tc; Tetracycline.

in Table 2, pHZ22 and pHZT224 could be mobilized into *Z. mobilis* ATCC31812 as well as *E. coli* HB101 with a help of RP4.

Expression of antibiotic resistant genes in *Z. mobilis*

The expression of chloramphenicol and tetracycline resistant genes in the host *Zymomonas* cells harboring pHZ22 and pHZT224 was tested (Table 3). For the comparison purpose, *E. coli* containing these plasmids and *Z. mobilis* without these were also tested with the varying concentrations of antibiotics. The minimal inhibitory concentration of chloramphenicol to *Z. mobilis* containing pHZ22 ranged from 200 to 300 μg/m*l*; *Z. mobilis* containing no plasmid could resist chloramphenicol in the concentrations between 50 and 80 μg/m*l*. *Z. mobilis* cells harboring pHZT224 also exhibited strong resistance against tetracycline with the concentrations up to 30 μg/m*l*. Without this plasmid, *Z. mobilis* could only survive under the condition of a low tetracycline concentration (5 μg/m*l*).

Table 3. Antibiotic resistance of host strains harboring plasmids.

Host strains	Chloramphenicol (μg/m*l*)					Tetracycline (μg/m*l*)				
	50	80	100	200	300	5	10	15	20	30
E. coli *C600* (pHZ22)	G[a]	G	G	G	G	N[b]	N	N	N	N
Z. mobilis ATCC31812 (pHZ22)	G	G	G	G	G	N	N	N	N	N
Z. mobilis ATCC31812	G	N	N	N	N	N	N	N	N	N
E. coli C 600 (pHZT224)	G	G	G	G	G	G	G	G	G	G
Z. mobilis ATCC31812 (pHZT224)	G	G	G	G	N	G	G	G	G	G
Z. mobilis ATCC31812	G	N	N	N	N	G	N	N	N	N

[a]: G represents growth [b]: N represents no growth

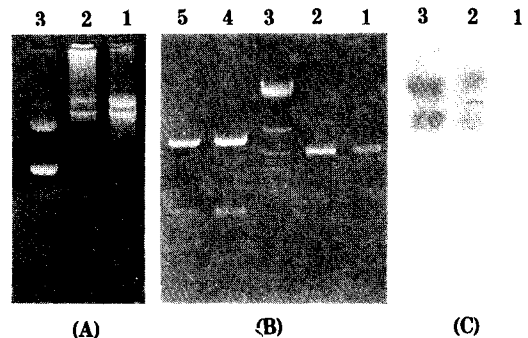

Fig. 7. Re-isolation and characterization of hybrid plasmid pHZ22.
(A); pHZ22 as a control
Lanes 1; natural plasmids from *Z. mobilis* ATCC31812, 2; pHZ22 from *Z. mobilis* ATCC31812, 3; pHZ22 from *E. coli* HB101
(B); Back-transformed pHZ22 into *E. coli* HB101
Lanes 1; pHZ22 from *E. coli* HB101 2; back-transformed pHZ22, 3; λ cI857 S7 DNA digested with AvaI, 4; 1 digested with HindIII, 5; 2 digested with HindIII
(C); DNA-DNA hybridization
Lanes 1; natural plasmids from *Z. mobilis* ATCC31812, 2; pHZ22 from *Z. mobilis* ATCC31812, 3; pHZ22 from *E. coli* HB101

Re-isolation and characterization of plasmids pHZ22 and pHZT224

The plasmids pHZ22 and pHZT224 were re-isolated from the host strain and characterized. In Fig. 7 (A) and Fig. 8 (A), identical electrophoretic patterns of the plasmid DNAs isolated from conjugant *Z. mobilis* ATCC31812 and *E. coli* HB101 are shown. These plasmids were back-transformed into *E. coli* HB101 and again isolated from back-transformant cells and digested with HindIII. Electrophoretic patterns in agarose gels exhibited identical fragments of these plasmids compared with those from the control strain of *E. coli* HB101 (Fig. 7(B) and Fig. 8(B)). Southern blot hybridization was also performed to confirm the identity of these plasmids. A small fragment (2.4 Kb) of pHZ22 digested with HindIII was used as a probe for these experiments. The probe was isotopically labelled with γ-(^{32}P)dATP by kination. Fig. 7(C) and Fig. 8(C) respectively represent identical autoradiographic images of conjugant plasmids from *Z. mobilis* ATCC31812 and plasmids from the control *E. coli* HB101 strain.

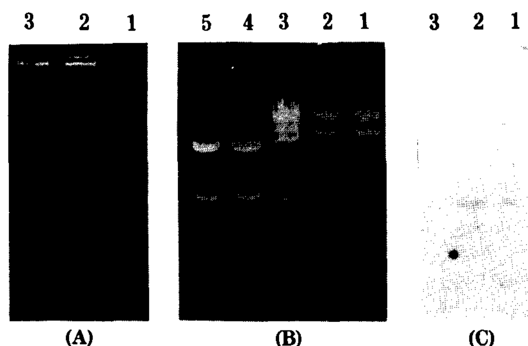

Fig. 8. Re-isolation and characterization of hybrid plasmid pHZT224.
(A); pHZT as a control
Lanes 1; pHZT224 from *E. coli* HB101, 2; pHZT224 from *Z. mobilis* ATCC31812, 3; natural plasmids from *Z. mobilis* ATCC31812
(B); Back-transformed pHZT224 into *E. coli* HB101
Lanes 1; pHZT224 from *E. coli,* 2; back-transformed pHZ224, 3; λ cI857 S7 DNA digested with AvaI, 4; 1 digested with HindIII, 5; 2 digested with HindIII
(C); DNA-DNA hybridization
Lanes 1; pHZT224 from *E. coli* HB101, 2; pHZT224 from *Z. mobilis* ATCC31812, 3; natural plasmids from *Z. mobilis* ATCC31812

Discussion

We have isolated five natural plasmids in different sizes from the *Z. mobilis* ATCC10988 strain. The numbers and sizes of plasmids from the same strain, however, have not always been agreed with each other in a number of investigations (12, 17, 18, 19). It was postulated through nick-translation and Southern blotting experiments that some plasmids from one species were homologous with each other (20). In favor of its comparatively small size (3.9 Kb) and suitable cleavage sites for restriction enzymes (Fig. 3), pZM3 was selected as a source of replication origin of *Z. mobilis* for the vector construction although no phenotype had yet been assigned. The *Z. mobilis* strain ATCC31821 was chosen as a host strain for this study. This was in respect of the least number of natural plasmids existing in the strain as well as the superiority of this strain to other *Zymomonas* strains for ethanol production (8).

Prior to constructing artificial vectors, a physical map of pZM3 was made. Since copy numbers of pZM3 in *Z. mobilis* are smaller than in *E. coli,* a hybrid plasmid containing full length of pZM3 and pBR325 was first constructed to obtain enough copy numbers from *E. coli* cells. This plasmid was digested with several restriction enzymes recognizing hexanucleotide sequences. A standard curve for the relationship between the mobility and the logarithm of molecular size (Kb) was prepared with fragments of λ cI 857 S7 DNA digested with Hind III and a 1 Kb ladder as standard size markers. As the linear range of the standard curve is changeable with the percentage of agarose content, 0.8% and 1.5% agarose gel electrophoresis were applied in order for restriction fragments of pZM3 to be included in the linear range of the standard curve. From this cruve, the fragment sizes of pZM3 were calculated and the final restricition map was constructed (Fig. 3).

Although *Zymomonas* strains were found to be resistant to a large number of antibiotics (Table 1), none of these properties appeared to be plasmid encoded. The resistance is perhaps rather due to the lack of uptake system of antibiotics in *Zymomonas*

cells. It was suggested that resistance to aminoglycoside in many anaerobic bacteria resulted from an inadequate electrical potential across the membrane to drive aminoglycoside uptake (1, 20). This mechanism of resistance may be acting in *Zymomonas* and imply that the resistant characteristics are of limited use in genetic studies of *Zymomonas*. The sensitivity of *Zymomonas* strains to tetracycline and chloramphenicol, however, potentially allowed us for direct selection of plasmids containing resistant genes for these antibiotics.

For the plasmid construction, pHZ22 was first made between pBR325 and pZM3. pBR325 was employed in order to exploit chloramphenicol resistant gene of this vector originating from transposon (21). HindIII was used for restriction as pZM3 had two restriction sites for this enzyme; pBR325 had one in the region of tetracycline resistant gene. Insertion of pZM3 digested with HindIII into the HindIII site of pBR325 destroyed the tetracycline resistant gene. However, this facilitated the selection of recombinant cells by use of $Cm^r Tet^s$ properties. Subsequently, trials were made to introduce tetracycline resistant gene to pHZ22. Since a broad host range plasmid RP4 contained the tetracycline resistant gene, this plasmid was chosen for this study. From the sequence analysis of RP4 (22), it was found that the total gene of tetracycline resistance was contained in the 2.4 Kb fragment and could be generated by double digestion with BglII and SmaI. Since the 2.4 Kb fragment has one cleavage site for SmaI, RP4 was digestcd with BglII first and then partially digested with SmaI. The optimum treating time for partial digestion was found to be 1 min and the sizes of fragments obtained were 24.3, 18.9, 9.5, 6.5, 2.5 and 1.7 Kb, respectively (data not shown).

Transmission of the newly constructed plasmids into host cells is indispensible in order to make these plasmids viable. However, it has been claimed that the conventional transformation methods using competent cells do not work in *Zymomonas* cells due to the different phospholipid and fatty acid composition of the cellular membrane from *E. coli* (23). In lieu of transformation, studies have shown that plasmids having a broad host range could be conjugally transferred into *Zymomonas* and stably maintained (7,8,12). In the present investigation, it was also not successful to transfer pHZ22 and pHZT224 into *Zymomonas* by transformation. As a consequence, conjugation was tried for the transfer. A helper plasmid was necessary for these plasmids to mobilize into recipient cells. Among helper plasmids such as RP4, pRK2013, pGC91.14 and R68, a common feature of which is *tra* gene (21), RP4 was chosen as a helper for this study. As shown in Table 2, it was evident that pHZ22 and pHZT224 could be mobilized into host cells with a help of RP4. The plasmid pBR325 could also be mobilized into *Z. mobilis* ATCC31812 but could not be maintained stably indicating that the replication origin of pBR325 from *E. coli* did not act in *Z. mobilis*. If pHZT224 conjugally transferred with a help of RP4, conjugant cells harboring pHZT224 might also contain RP4. In this case, tetracycline resistant gene might have been expressed by not only pHZT224 but also RP4. To avoid this situation, conjugant cells were reselected from plates containing chloramphenicol (30 μg/ml) and tetracycline (12 μg/ml) after the primary selection from plates containing chloramphenicol (30 μg/ml) and streptomycin (80 μg/ml). Streptomycin was used since recipient cells (*E. coli* HB101 and *Z. mobilis* ATCC31812) were considerably resistant to this antibiotics whereas donor cells (*E. coli* C600) were not. The antibiotic resistance of *Z. mobilis* ATCC31812 harboring pHZ22 and pHZT224 against chloramphenicol and tetracycline (Table 3) indicated that these antibiotic resistant genes could be expressed in *Z. mobilis* to a considerable level. This fact strongly suggests that the promoters of the antibiotic resistant genes could likely be used for the expression of other useful genes to be cloned in *Z. mobilis*.

In order to show the evidence that the plasmids pHZ22 and pHZT224 were conjugally transferred into *Z. mobilis* ATCC31812 and could replicate autonomously in the host, these plasmids were reisolated and characterized. The comparison of lanes 2 and lanes 3 in Fig. 7(A) and Fig. 8(A) indicated that these respective two supercoil DNAs were the same in size. Since *Z. mobilis* ATCC31812 contained ill-

characterized native plasmids, plasmid DNA mixture from conjugants was back-transformed into *E. coli* HB101 containing no plasmids. Plasmid DNAs from these backtransformants were again isolated and digested with HindIII. The comparison of lanes 3 and lanes 4 in Fig. 7(B) and Fig. 8(B) clearly indicated that these plasmids were respectively identical. This confirmation was further supported by Southern blot hybridization of plasmids. After hybridization with the probe DNAs, DNAs were found to be homologous with the probe DNAs (Fig. 7(C) and Fig. 8(C)) indicating that the DNAs were identical. From all these results, it was evident that both pHZ22 and pHZT224 could be replicable in *Z. mobilis* ATCC31812 with the pZM3 replication origin undisrupted during the processes of the vector construction.

To determine the stabilities of pHZ22 and pHZT224 in *Z. mobilis* ATCC31812, the organism was cultured for 24 hrs in the selective (RM plus 30 $\mu g/ml$ chloramphenicol) and non-selective (RM) media. By plating the cells on RM plates, the proportion of plasmid-carrying cells was determined against the total cells grown. It was found that pHZ22 exhibited 93% of stability regardless of medium composition over 30 generations while pHZT224 showed 97% of stability (25).

In conclusion, the plasmid vectors, pHZ22 and pHZT224, developed in the present investigation have distinctive characteristics as cloning vehicles for *Z. mobilis*. The plasmids have the replication origin of *Z. mobilis* which is functional in both *Z. mobilis* and *E. coli*. They have antibiotic resistant gene markers for chloramphenicol (pHZ22) as well as tetracycline (pHZT224). The sizes of the plasmids are 8.4 and 10.7 Kb, respectively, to be considerably small. They are also transmissible into host cells by conjugation and stably maintained by replicating autonomously. With all these characteristics, it was evident that the plasmids developed in this study could be used as useful cloning vectors to introduce foreign genes such as amylase or cellulase genes into *Zymomonas* cells.

요 약

알코올 생산성이 높은 *Zymomonas* 균주의 기질 이용성을 넓히기 위한 목적으로 natural replicon을 포함하며 적당한 항생제 저항표지를 갖는 plasmid vector의 제조를 시도하였다.

Z. mobilis ATCC10988에서 분리된 몇개의 plasmid중 3.9 kb의 적당한 크기를 갖는 pZM 3를 선정하여 수종의 제한효소로 처리하여 절편의 크기에 따라 유전자 지도를 작성하였다.

pZM 3의 replicon과 pBR 325의 chloramphenicol 저항유전자를 포함한 재조합 plasmid인 pHZ 22를 개발하고 이 plasmid vector가 숙주세포인 *Z. mobilis* ATCC31821에서 독립적으로 replication됨을 확인하였다.

또 하나의 항생제 저항표지로서 RP 4의 tetracycline 저항유전자를 분리하여 pHZ 22에 도입함으로써 pHZT 224를 제조하였는데 이 plasmid vector도 *Zymomonas*로 conjugation에 의해 전이되어 안정하게 유지되었다.

본 연구를 통하여 개발된 plasmid vector는 *Z. mobilis*와 *E. coli*에 공히 작용하는 shuttle vector로서 외부 유전자를 *Zymomonas*에 도입시킬 수 있는 유용한 유전자 운반체임이 확인되었다.

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