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Expression and Stability of a Recombinant Plasmid in *Zymomonas mobilis* and *Escherichia coli*

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A recombinant plasmid was constructed by ligating *Eco*RI digests of the plasmid cloning vector pBR325 and pZMO2, one of the natural plasmids of *Zymomonas mobilis* ATCC 10988. This vector, named pDS212 (total size 7·9 kb), which was able to transform *Escherichia coli* efficiently, was also transferred to *Z. mobilis* hosts by mobilization during conjugation using the helper plasmid pRK2013. pDS212 was inherited stably in both *E. coli* and *Z. mobilis* hosts and could be recovered intact from them. Markers of pBR325 and pRK2013 were also transferred in *Z. mobilis* but at very low frequencies. Neither pBR325 nor pRK2013 could be recovered intact from the *Z. mobilis* hosts. It is proposed that expression and stability of pDS212 in *Z. mobilis* is due to the origin of replication of pZMO2 that it carries, and that it may be used for developing a gene transfer system in *Z. mobilis*.

INTRODUCTION

Recent studies have shown that the Gram-negative anaerobic bacterium *Zymomonas mobilis* is a potential ethanol-producing micro-organism which has a number of advantages in ethanol fermentations compared to traditional yeasts (Rogers *et al.*, 1984; Bringer *et al.*, 1984; Doelle & Greenfield, 1985*a*, *b*). However, the use of *Z. mobilis* in industrial ethanol fermentations is limited, because it can efficiently ferment only glucose and fructose (Swings & De Ley, 1977). Sucrose may also be fermented but with a lower yield of ethanol, because of the formation of sorbitol and levan (Viikari, 1984*a*, *b*; Viikari & Linko, 1986).

In spite of the considerable amount of information on the kinetics and biochemistry of *Z. mobilis* ethanol fermentations, very little is known about the genetics of the organism. Skotnicki *et al.* (1980) first reported transfer of R-plasmids to *Z. mobilis* by inter-species conjugation. This system was used successfully by Carey *et al.* (1983) to transfer and express a lactose transposon in *Z. mobilis*. Transformation of *Z. mobilis* by an *in vitro* cointegrate of a *Z. mobilis* natural plasmid with an *Escherichia coli* plasmid (total size 28 kb) has also been reported (Browne *et al.*, 1984). However, there are certain obstacles to the utilization of these plasmids as cloning vectors for *Z. mobilis*, such as their large size and unknown stability within the host cells. Plasmid molecules have been detected in various *Z. mobilis* strains (Dally *et al.*, 1982; Tonomura *et al.*, 1982; Drainas *et al.*, 1983). These molecules may be used in whole or in part for the construction of suitable cloning vectors. Such a potential vector was constructed by ligating the plasmid pZMO2 of *Z. mobilis* ATCC 10988 (Drainas *et al.*, 1983) in the *Eco*RI restriction site of the plasmid vector pBR325. In the present report the transfer, expression and stability of the above recombinant plasmid in *Z. mobilis* are described. It is proposed that this plasmid may be used as a suitable shuttle cloning vector for the development of a high-efficiency gene-transfer system in *Z. mobilis*.

METHODS

Growth of cultures. E. coli strains were grown in Luria media as described by Maniatis *et al.* (1982). *Z. mobilis* strains were grown in a complete liquid medium (*Zymomonas* broth) containing (w/v) 0·1% KH_2PO_4, 0·1%

Table 1. *Bacterial strains and plasmids*

Species	Strain	Plasmid	Plasmid marker	Source*	Reference
E. coli	RR1	—	—	J.R.K.	Bolivar *et al.* ((1977)
	RR1	pBR325	Ap^R Tc^R Cm^R	J.R.K.	Bolivar (1979)
	RR1	pDS212	Ap^R Tc^R	–	Present report
	HB101	pRK2013	Km^R *tra*$^+$	K.L.	Friedman *et al.* (1982)
	RR1	pRK2013 + pBR325	Km^R Ap^R Cm^R Tc^R *tra*$^+$	–	Present report
	RR1	pRK2013 + pDS212	Km^R Ap^R Tc^R *tra*$^+$	–	Present report
Z. mobilis	ATCC 10988	pZMO1,2,3,4,5,6	ND	W.M.L.	Drainas *et al.*(1983)
	CU1	pZMO1,2,3,5,6	ND	–	Drainas *et al.* (1984)
	CU1-Rif2	pZMO1,2,3,5,6	ND	–	Present report
	CP4	pRUT41	Gm^R Km^R Str^R	W.M.L.	Walia *et al.* (1984)
	NCIB 11163	ND	ND	NCIB	—

ND, Not defined.

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$(NH_4)_2SO_4$, 0·05% $MgSO_4$, 0·5% yeast extract (Oxoid) and 2% glucose. Liquid cultures were incubated at 30 °C without agitation. For solid media 2% (w/v) agar (Serva) was added (*Zymomonas* agar). Kanamycin (100 μg ml^{-1}), rifampicin (10 μg ml^{-1}) or tetracycline (40 μg ml^{-1}) were added to liquid or solid media as specified in the text. All *Z. mobilis* strains tested were sensitive on media containing over 50 μg kanamycin ml^{-1}, 5 μg rifampicin ml^{-1} or 10 μg tetracycline ml^{-1}, and resistant on 100 μg ampicillin ml^{-1}.

Strains and plasmids. These are summarized in Table 1. *E. coli* RR1 derivatives containing both pBR325 and pRK2013 [RR1(pRK2013 + pBR325)] or pDS212 and pRK2013 [RR1(pRK2013 + pDS212)] were constructed by conjugation. pRK2013 is a large recombinant plasmid containing the Tra^+ functions of the conjugative plasmid RK2 and a gene marker coding for resistance to kanamycin (Friedman *et al.*, 1982). *Z. mobilis* CU1-Rif2 (resistant to 10 μg rifampicin ml^{-1}) was isolated from CU1 after acridine orange treatment. For this purpose 10^8 cells from a late-exponential phase culture were inoculated into 10 ml *Zymomonas* broth containing 100 μg acridine orange ml^{-1}, and the suspension was incubated at 30 °C for 10 h. Rifampicin-resistant colonies were isolated after direct plating on *Zymomonas* agar containing 10 μg rifampicin ml^{-1}.

Chemicals. Agarose, sodium dodecyl sulphate, caesium chloride, restriction endonucleases and T4 DNA ligase were from Bethesda Research Laboratories. DNA from phage P22 was kindly provided by Dr Marc Orbach (Stanford University, Calif., USA). Antibiotics were from Sigma. All other chemicals were from Serva.

Isolation and analysis of plasmids. Mini-screen tests for plasmid detection were as described by Drainas *et al.* (1984). Plasmids from *E. coli* strains were extracted and purified by CsCl density gradient ultracentrifugation as described by Maniatis *et al.* (1982). Plasmid DNA was analysed by horizontal agarose gel electrophoresis as described by Meyers *et al.* (1976).

Recombinant DNA procedures. Restriction digestions and ligations were done by standard methods (Maniatis *et al.*, 1982). Plasmid pBR325 (Bolivar, 1979) was used as the cloning vector. Recombinant plasmids were isolated by direct transformation of *E. coli* RR1 as described by Cohen *et al.* (1972).

Conjugation. Filter matings on Sartorius nitrocellulose filters (0·45 μm pore size, 25 mm diameter) were done by standard methods with minor modifications (Skotnicki *et al.*, 1980). Samples of late-exponential cultures of donor and recipient strains were added to the filter at a donor : recipient ratio of 2 : 1. The filter was then placed on the surface of a solid medium plate and incubated at 30 or 37 °C (using *Z. mobilis* or *E. coli* as recipient, respectively) for 5 h. The cells were resuspended in liquid medium and plated on selective media for the isolation of transconjugants. *E. coli* RR1 transconjugants carrying both pRK2013 and pBR325 were isolated on Luria agar containing tetracycline and kanamycin (20 μg ml^{-1} each) by mating HB101(pRK2013) (donor) with RR1(pBR325) (recipient). *E. coli* RR1 transconjugants carrying both pRK2013 and pDS212 were isolated as above using RR1(pDS212) as the recipient.

Plasmid stability. This was estimated by measuring the stable inheritance of the antibiotic resistance plasmid markers. The percentage of Tc^R (for *Z. mobilis*) or Tc^R and Ap^R (for *E. coli*) colonies per cell division on non-selective conditions (agar medium without antibiotic) was determined (Kieser *et al.*, 1982).

RESULTS AND DISCUSSION

Construction and restriction map of the recombinant plasmid pDS212

We have previously shown that the natural plasmid pZMO2 of *Z. mobilis* ATCC 10988 (1·9 kb: Drainas *et al.*, 1983) contains single sites for the restriction endonucleases *Eco*RI and

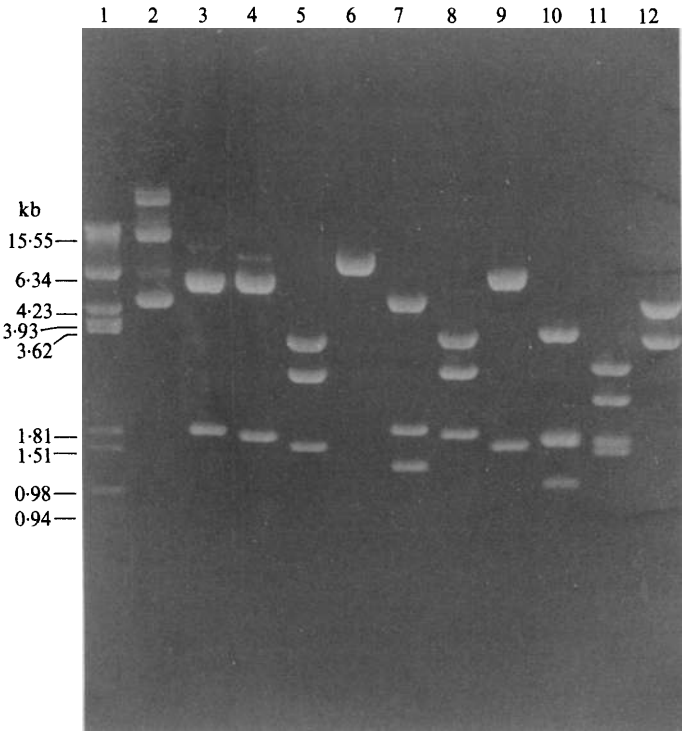

Fig. 1. Agarose gel electrophoresis of restriction fragments of pDS212. Lanes: (1) P22 DNA digested with *Hin*dIII; (2) pDS212 not digested (front band, ccc; back bands, oc); (3–12) pDS212 digested with restriction endonucleases: (3) *Eco*RI, (4) *Eco*RV, (5) *Pvu*II, (6) *Hin*dIII, *Bam*HI, or *Pst*I (linearized form), (7) *Eco*RI + *Hin*dIII, (8) *Pst*I + *Eco*RV, (9) *Eco*RV + *Hin*dIII, (10) *Pvu*II + PstI, (11) *Pvu*II + *Hin*dIII, (12) *Hin*cII.

*Eco*RV and two sites for *Pvu*II (A. Scordaki & C. Drainas, unpublished results). Therefore, a ligate of pZMO2 and pBR325, linearized by *Eco*RI digestions, was used to transform *E. coli* RR1. Recombinant transformants were selected for resistance to ampicillin and tetracycline, and for sensitivity to chloramphenicol. Plasmids from three recombinant transformants were isolated and purified by CsCl density gradient ultracentrifugation. Linear fragments produced by single and double digestions with various endonucleases were analysed by agarose gel electrophoresis (Fig. 1). The restriction maps of all the recombinant plasmids paralleled those of the parental plasmid molecules (Fig. 2). All the recombinant plasmids had a total size equivalent to 5·98 + 1·9 kb. One of these, named pDS212, was tested for expression and stability in *Z. mobilis* and *E. coli*.

Stability of pDS212 in E. coli

The recombinant plasmid pDS212 transformed *E. coli* RR1 at frequencies similar to pBR325 (1×10^5 and $3{\cdot}3 \times 10^5$ transformants per μg DNA respectively). Six isolates picked randomly from among these transformants were tested for plasmid stability. All maintained both the gene markers of pDS212 at a frequency of 98% after 60 cell divisions under non-selective conditions (Table 2).

Transfer, expression and stability of pDS212 in Z. mobilis

Efforts were made to transform the *Z. mobilis* strains NCIB 11163, CP4 and CU1-Rif2 with either pBR325 or pDS212. Methods previously described to be successful for *Z. mobilis* (Browne

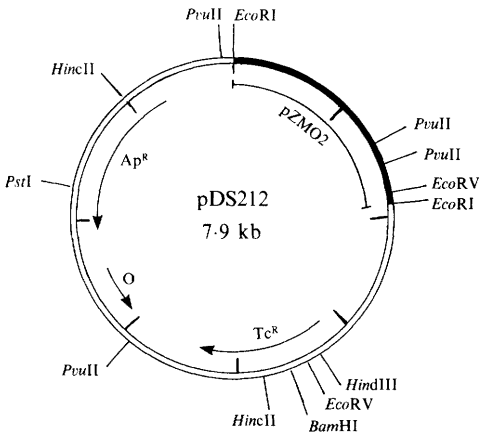

Fig. 2. Restriction map of pDS212 (one division ≃ 1 kb).

Table 2. *Stability of pDS212 in E. coli and Z. mobilis*

		Plasmid stability*						
		No. of cell divisions:						
Strain	Plasmid	0	10	20	30	40	50	60
RR1	pBR325	100	100	100	100	100	100	100
RR1	pDS212	100	100	100	100	100	99	98
CU1-Rif2	pDS212	100	100	99	98	97	95	93

* Estimated as the percentage of colonies expressing the plasmid markers after growth on non-selective conditions (500 colonies were tested).

et al., 1984) were used without success. Tc^R colonies were obtained at low frequencies ($\sim 3 \times 10^{-6}$ transformants per μg DNA), but pDS212 was not recovered either on plasmid mini-screens or by back-transformation in *E. coli*. It was therefore concluded that resistance to tetracycline could be due to the spontaneous mutation frequency, under the conditions of the experiment. Conjugative plasmids like pRK2013 (Friedman *et al.*, 1982) are known to mobilize other non-conjugative plasmids during conjugation. Although pDS212 is not a conjugative plasmid, its transfer in *Z. mobilis* was accomplished by this method using CU1-Rif2 as recipient and *E. coli* RR1(pRK2013 + pDS212) as donor. The transfer frequency was approximately 10^{-4} transconjugants per recipient (Table 3). Selection of transconjugants was made after 7 d incubation at 30 °C on *Z. mobilis* agar medium containing 40 μg tetracycline ml^{-1} (Table 3). Liquid growth tests showed that these transconjugants could grow in the presence of up to 60 μg tetracycline ml^{-1}.

E. coli RR1(pRK2013 + pBR325) donors were used to test possible transfer and expression of the vector pBR325 in CU1-Rif2 recipients. Tc^R putative transconjugants were again obtained but at very low frequency (Table 3). These transconjugants appeared 2 d later on the selective medium than did those described above, and they were not able to grow in the presence of over 20 μg tetracycline ml^{-1} in liquid medium, an indication that the transferred marker was present at lower copy number than the marker of pDS212. Selection on 100 μg kanamycin ml^{-1} showed transfer of the Km^R marker of the conjugative plasmid pRK2013 at low frequency when either donor was used (Table 3). Transconjugants on selective media containing both tetracycline and kanamycin were not obtained.

Six Tc^R and Km^R isolates were picked at random among the *Z. mobilis* transconjugants from each of the above conjugations and were tested by the mini-screen method for occurrence of

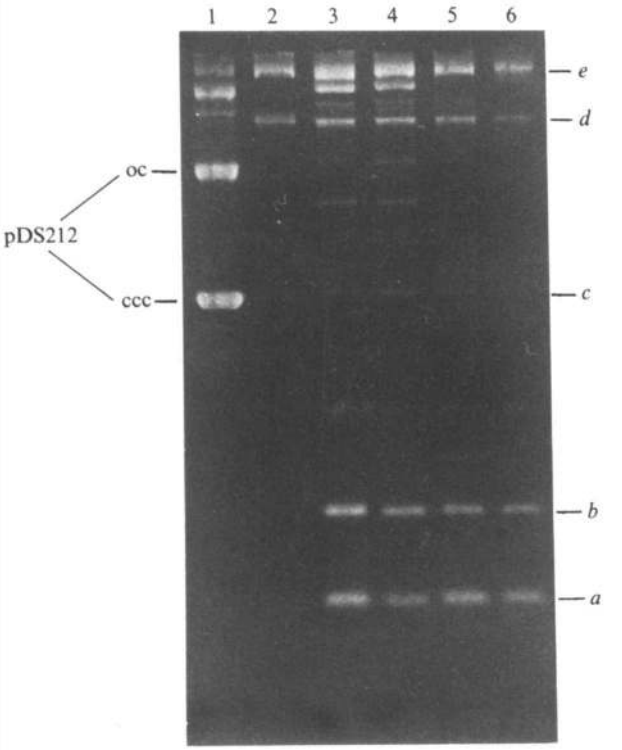

Fig. 3. Mini-screen test for plasmids of *Z. mobilis* transconjugants. Lanes: (1) RR1(pDS212), (2) HB101(pRK2013), (3) tcj 1, (4) tcj 2, (5) tcj 3, (6) CU1-Rif2. tcj, *Z. mobilis* transconjugants containing pDS212 (tcj 4, 5, 6 were similar to tcj 1, 2, 3 and are omitted for clarity); *a*, *b*, *c*, *d*, *e*, *Z. mobilis* natural plasmids.

Table 3. *Mobilization of pDS212 in CU1-Rif2 by conjugation*

No.	Donor (2×10^8)	Recipient (1×10^8)	Frequency of transconjugants*	
			Rif + Tc	Rif + Km
1	RR1(pRK2013 + pDS212)	CU1-Rif2	1×10^{-4}	3×10^{-7}
2	RR1(pRK2013 + pBR325)	CU1-Rif2	$3{\cdot}5 \times 10^{-7}$	$3{\cdot}5 \times 10^{-7}$
3	RR1(pBR325)	CU1-Rif2	$<10^{-8}$	$<10^{-8}$
4	RR1(pDS212)	CU1-Rif2	$<10^{-8}$	$<10^{-8}$
5	RR1(pRK2013 + pDS212)	—	$<10^{-8}$	$<10^{-8}$
6	RR1(pRK2013 + pBR325)	—	$<10^{-8}$	$<10^{-8}$
7	—	CU1-Rif2	$<10^{-8}$	$<10^{-8}$

* The values are the means of six repeats. The selective conditions were either 10 μg Rif ml^{-1} + 40 μg Tc ml^{-1} or 10 μg Rif ml^{-1} + 100 μg Km ml^{-1},

transferred plasmid molecules. The plasmid profiles obtained by agarose gel electrophoresis of extracts of *Z. mobilis* transconjugants from conjugation no. 1 (where pDS212 was the mobilized plasmid: Table 3) revealed a band corresponding to the open circle of plasmid pDS212 (Fig. 3). This band was absent from the recipient CU1-Rif2. It would be expected that the native plasmid pZMO2 should not be maintained together with pDS212, because they share a common origin of replication. This cannot be been in Fig. 3, because it has been proved that plasmid pZMO2 co-

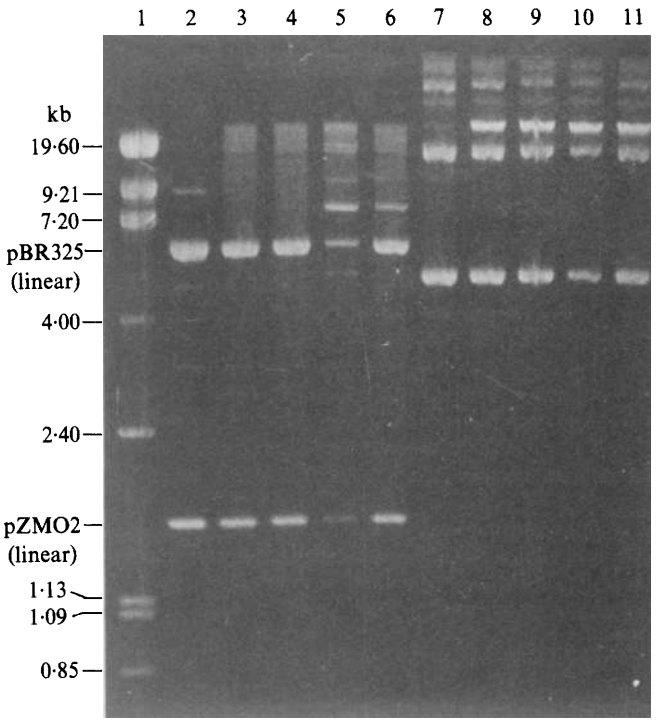

Fig. 4. Structural analysis of pDS212 isolated from *Z. mobilis* hosts and transferred into *E. coli*. Lanes: (1) P22 DNA digested with *Eco*RI; (2) pDS212 purified on a CsCl density gradient digested with *Eco*RI; (3) mini-screen plasmid extract of RR1(pDS212) digested with *Eco*RI; (4), (5), (6) mini-screen plasmid extracts of RR1 transformed by extracts of tcj 1, 2 and 3, respectively, digested with *Eco*RI; (7)–(11) as for (2)–(6), respectively, but not digested.

migrates with pZMO1 on agarose gels (A. Scordaki & C. Drainas, unpublished results). Therefore, band (*a*) in Fig. 3 should represent pZMO1. The plasmid profiles of the Km^R and the Tc^R transconjugants from conjugation no. 2 (where pBR325 was the mobilized plasmid: Table 3) were identical to the profile of CU1-Rif2 and for clarity are omitted from Fig. 3.

The plasmid extracts of the transconjugants from both conjugations were used to transform *E. coli* RR1. Extracts of transconjugants from conjugation no. 1 transformed RR1 for both markers of pDS212 (Table 4). Plasmid profiles of extracts from these transformants showed the bands of pDS212. A restriction digest of the extracts with *Eco*RI recovered the linear fragments corresponding to pBR325 and pDS212 (Fig. 4). Three randomly selected *Z. mobilis* CU1-Rif2 transconjugants carrying pDS212 were grown on non-selective liquid medium to test plasmid stability. The Tc^R marker was maintained at a frequency of 93% after 60 cell divisions under non-selective conditions (Table 2). Three Tc^R transconjugants isolated at random after 60 cell divisions under selective or non-selective conditions respectively showed the same plasmid stability and integrity for pDS212 as above (results not shown). These results strongly indicate that pDS212 was transferred and expressed stably in *Z. mobilis* cells as an intact plasmid molecule and that it remained as such after many rounds of replication.

Plasmid extracts of Tc^R transconjugants from conjugation no. 2 (Table 3) transformed RR1 for Tc^R at a frequency 1000-fold lower than the above (Table 4). None of these transformants carried all the markers of pBR325. Restriction analysis of plasmid DNA isolated from both Ap^S and Cm^S transformants had a different pattern from either pBR325 or pDS212. These results indicate that pBR325 undergoes genetic rearrangement within the *Z. mobilis* hosts. This rearrangement did not lead to spontaneous *in vivo* formation of a recombinant plasmid molecule

Table 4. *Transformation of E. coli RR1 with plasmid extracts from Z. mobilis transconjugants*

Transconjugant plasmid extract*	Frequency of transformation†			
	Ap	Tc	Cm	Km
tcj 1	$3{\cdot}8 \times 10^{-3}$	$3{\cdot}8 \times 10^{-3}$	–	–
tcj 2	$3{\cdot}5 \times 10^{-3}$	$3{\cdot}5 \times 10^{-3}$	–	–
tcj 3	$2{\cdot}8 \times 10^{-3}$	$2{\cdot}8 \times 10^{-3}$	–	–
tcj 4	$2{\cdot}5 \times 10^{-3}$	$2{\cdot}5 \times 10^{-3}$	–	–
tcj 5	$4{\cdot}9 \times 10^{-3}$	$4{\cdot}9 \times 10^{-3}$	–	–
tcj 6	$4{\cdot}3 \times 10^{-3}$	$4{\cdot}3 \times 10^{-3}$	–	–
tcj 11	–	–	–	$2{\cdot}5 \times 10^{-5}$
tcj 12	–	–	–	$2{\cdot}1 \times 10^{-5}$
tcj 13	–	–	–	$2{\cdot}3 \times 10^{-5}$
tcj 27	$4{\cdot}1 \times 10^{-6}$	$4{\cdot}1 \times 10^{-6}$	–	–
tcj 28	–	$4{\cdot}7 \times 10^{-6}$	$4{\cdot}7 \times 10^{-6}$	–
tcj 29	–	$3{\cdot}8 \times 10^{-6}$	$3{\cdot}8 \times 10^{-6}$	–

* tcj 1–6: CU1-Rif2 Tc^R transconjugants from mobilization of pDS212; tcj 11–13: CU1-Rif2 Km^R transconjugants from mobilization of pDS212 and pBR325; tcj 27–29: CU1-Rif2 Tc^R transconjugants from mobilization of pBR325.

† No. of transformants per recipient ($\sim 10^8$ recipient *E. coli* RR1 cells were used per transformation). The frequency of spontaneous resistance was $< 1 \times 10^{-8}$ in all cases.

equivalent to pDS212. Plasmid extracts of Km^R *Z. mobilis* transconjugants were also able to transform RR1 for Km^R, but again at a low frequency (Table 4). Restriction analysis of the transforming plasmids gave a different pattern from the restriction of pRK2013 and it was again concluded that the helper plasmid also undergoes genetic rearrangement within the *Z. mobilis* hosts. Study of the molecular events following these genetic rearrangements is in progress.

General conclusions

The recombinant plasmid pDS212 derived from the ligation of pBR325 and pZMO2, which transformed *E. coli* efficiently, was also transferred, replicated and expressed stably in *Z. mobilis*. In contrast, the parental plasmid vector pBR325 and the helper conjugative plasmid pRK2013 were transferred to *Z. mobilis* hosts at very low frequencies and failed to express stably in the same hosts. The above evidence supports the theory that the expression and stability of pDS212 in *Z. mobilis* and *E. coli* is due to the origins of replication for both organisms that it carries. The recombinant plasmid pDS212 has a number of advantages as a potential cloning vector for developing a gene transfer system in *Z. mobilis*. These may be summarized as follows: (1) it has a small size (7·9 kb); (2) it carries two convenient gene markers with single restriction sites within them, suitable for cloning DNA fragments; (3) it can replicate and express stably in *Z. mobilis* and *E. coli*. The failure to transform *Z. mobilis* with pDS212 may be due to DNA uptake limitations or to a possible restriction system. Research on this problem is in progress.

Two reports describing cloning vectors for *Z. mobilis* appeared while this manuscript was being reviewed. In the first, cloning vectors were constructed by ligation of a 3·9 kb natural plasmid of *Z. mobilis* and plasmid pACYC184 (Tonomura *et al.*, 1986). All the constructed recombinant vectors had low stability in the *Z. mobilis* hosts (38–77% after about six cell divisions under non-selective conditions) and only one available marker (Tc^R or Cm^R) for cloning foreign DNA. In the second, a vector carrying a deletion derivative of pBR327, the lactose permease gene and a functional β-galactosidase fragment was proved suitable for isolating *Z. mobilis* promoters (Byun *et al.*, 1986). However, the vector has instability problems and apparently is larger than pDS212. In conclusion, for the reasons listed above, we propose that pDS212 is more advantageous for cloning in *Z. mobilis*. In particular, a recombination of pDS212 with *Z. mobilis* promoters, as speculated by Byun *et al.* (1986), may produce a stable and high-expression vector for developing an efficient gene transfer system in *Z. mobilis*.

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