APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1980, p. 7-12
0099-2240/80/00-0007/06$02.00/0
Vol. 40, No. 1

R-Plasmid Transfer in *Zymomonas mobilis*

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Conjugal transfer of three IncP1 plasmids and one IncFII plasmid into strains of the ethanol-producing bacterium *Zymomonas mobilis* was obtained. These plasmids were transferred at high frequencies from *Escherichia coli* and *Pseudomonas aeruginosa* into *Z. mobilis* and also between different *Z. mobilis* strains, using the membrane filter mating technique. Most of the plasmids were stably maintained in *Z. mobilis*, although there was some evidence of delayed marker expression. A low level of chromosomal gene transfer, mediated by plasmid R68.45, was detected between *Z. mobilis* strains. Genetic evidence suggesting that *Z. mobilis* may be more closely related to *E. coli* than to *Pseudomonas* or *Rhizobium* is discussed.

The bacterium *Zymomonas mobilis*, which is used in the tropics to make alcoholic palm wines (27, 28, 31), appears to have considerable potential for industrial alcohol fermentations. This organism, which uses the Entner-Doudoroff pathway for glucose catabolism (12, 31), can produce up to 1.9 mol of ethanol per mol of glucose fermented (13, 14, 31). Recent reports from this laboratory have established that *Z. mobilis* can ferment high concentrations of glucose rapidly to ethanol in both batch and continuous cultures, with higher specific glucose uptake rates and ethanol production rates than for yeasts currently used in alcohol fermentations (22, 29).

Z. mobilis does, however, lack certain properties which would be very useful if it were to be used industrially. For example, it can only ferment glucose, fructose, and sucrose and cannot utilize other carbon sources such as maltose or starch (31). Direct fermentation of starch by *Z. mobilis*, rather than enzymatic hydrolysis followed by fermentation, would be a more economical method of converting starchy substrates such as cassava into alcohol.

Although it may be possible to genetically manipulate *Z. mobilis* to ferment starch, no methods for genetic analysis of this organism have so far been described. For this reason, the possibility of introducing transferable drug resistance plasmids was investigated in *Z. mobilis*. Experiments were designed to determine the ability of *Z. mobilis* to receive and transfer deoxyribonucleic acid by conjugation and to test for R-plasmid-mediated transfer of chromosomal genes between different strains of *Z. mobilis*.

Because *Zymomonas* is considered to be taxonomically related to *Pseudomonas* (6, 25, 31), derivatives of IncP1 plasmids which are readily transferable to *Pseudomonas* were used in these experiments. A derivative of plasmid RP4, pRD1 (which carries the *his nif* region of *Klebsiella pneumoniae*) (11), was used, as well as plasmid pJB4JI, which is derived from pPH1JI and carries Mu and transposon Tn*5* (2). A third P1 group plasmid used was R68.45, a variant of plasmid R68 selected for its high level of chromosome mobilization ability (16, 17). These three plasmids all confer multiple drug resistance and have a relatively wide host range among gram-negative bacteria (2, 17, 18, 23).

One other plasmid used in this study, R1*drd*19, is of a different incompatibility group from the other three plasmids, belonging instead to the IncFII group of plasmids. R1*drd*19 was tested because it can be transferred at high frequency between *Escherichia coli* strains and has been used to mediate chromosomal transfer between enterobacteria (7, 8, 26). This plasmid, however, is generally considered to have a more limited host range than IncP1 group plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids are described in Table 1.

Media. *Pseudomonas aeruginosa* and *E. coli* strains were grown in Luria broth with 5 g of glucose per liter (24). *Z. mobilis* strains were grown in RM medium, which contained, per liter, 20 g of glucose, 10 g of yeast extract (Oxoid), and 2 g of KH_2PO_4 (separately autoclaved). The pH was adjusted to 6.0, and 15 g of agar (Difco) per liter was added for solid media. The saline phosphate buffer used for dilution contained, per liter, 8.5 g of NaCl, 7 g of K_2HPO_4, and 3 g of KH_2PO_4, adjusted to pH 7.2.

Antibiotics. The different strains of *Z. mobilis*

TABLE 1. *Bacterial strains and plasmids used*

Bacteria and plasmids	Relevant properties and other strain no.	Reference
Bacteria		
Z. mobilis		
ZM1	ATCC 10988	31
ZM11	Spontaneous double mutant of ZM1 resistant to Sm and Rp, obtained in two steps	This paper
ZM4	CP4	31
ZM6	ATCC 29191, Z6	31
ZM61	Spontaneous double mutant of ZM6 resistant to Cm and Ap, obtained in two steps	This paper
E. coli		
SB1801	*his gnd str*	11
J53	*pro met nal*	9
P. aeruginosa		
PAO25	*arg leu*	16
Plasmids		
pRD1	RP4 Km Tc Cb, carrying the *his nif* region of *K. pneumoniae*	11
pJB4JI	pPH1JI Sp Gm Sm, carrying Mu and Tn*5* (Km)	2
R68.45	Km Tc Cb	16
R1*drd*19	Km Sm Cm Ap Su	9

used in this study were tested with increasing levels of each individual antibiotic to find the minimum concentrations which would completely inhibit growth. These concentrations were then used in all subsequent experiments. Strains ZM1 and ZM6 were found to have similar levels of resistance to the various drugs, whereas strain ZM4 was resistant to considerably higher levels of most of the drugs tested.

For conjugation experiments, RM medium was used with appropriate antibiotics at the following concentrations: ampicillin (Ap), 100 μg/ml; carbenicillin (Cb), 300 μg/ml; chloramphenicol (Cm), 12.5 μg/ml; kanamycin (Km), 20 μg/ml; rifampin (Rp), 50 μg/ml; spectinomycin (Sp), 20 μg/ml; streptomycin (Sm), 50 μg/ml; and tetracycline (Tc), 5 μg/ml. Where strain ZM4 was used as the recipient, concentrations of carbenicillin, kanamycin, and tetracycline were increased to 600, 50, and 10 μg/ml, respectively.

Conjugation experiments. Donor and recipient cultures were grown in liquid media at 30°C, and log-phase cells were mixed in the approximate ratio of three donor cells to one recipient cell. The mixtures were collected onto 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.) and incubated on RM plates at 30°C. The cells were then washed off the filters into saline phosphate buffer. After further dilution in buffer, the mixtures were plated on various selective and nonselective media. Plates were incubated at 30°C for up to 5 days to allow colony development. Transfer frequencies are expressed per recipient cell.

Z. mobilis has a very distinctive type of colony which is circular, convex, cream colored, and shiny and which is very different from colonies of *E. coli* or *P. aeruginosa*. This characteristic, together with any appropriate selective antibiotics, was sufficient to distinguish *Z. mobilis* transconjugants from donor strains in intergeneric matings.

Transfer of multiple drug resistance was determined by replica-plating colonies selected on a single antibiotic to media containing the other antibiotics.

Retransfer of plasmids between *Z. mobilis* strains was tested by both membrane filter matings and patch matings (4, 19). For patch crosses, loopfuls of donor and recipient cultures were mixed on RM plates and incubated at 30°C for 2 days before being replica-plated onto appropriate selective media. High-frequency transfer of a marker gave confluent growth on the selective media, whereas other crosses and controls only gave a few spontaneous drug-resistant mutants.

RESULTS

Growth medium for *Z. mobilis*. Although minimal media have been described for *Z. mobilis* (31), they do not support sustained, rapid growth of the strains used in this study. Also, the standard rich medium for *Z. mobilis* (31) was found to give low growth yields when cultures were grown aerobically in liquid medium. When solidified with agar, this rich medium only permitted about 10% of viable cells plated to form colonies under aerobic conditions. Therefore, to facilitate genetic analysis it was first necessary to devise a medium which would permit rapid growth of *Z. mobilis* in both liquid and solid media, with 100% colony formation.

The medium devised was RM, the composition of which is described in Materials and Methods. Marked variation in growth and colony formation was obtained depending on the brands of yeast extract and agar used. However, the combination of Oxoid yeast extract, Difco agar, and added phosphate was found to allow good growth and 100% colony-forming efficiency. This medium was then used for genetic analysis of *Z. mobilis*.

Optimal conditions for conjugation with *Z. mobilis*. Because conjugation between different bacterial genera has proved most successful with the membrane filter technique (4, 5, 32), this method was chosen to test for plasmid transfer to *Z. mobilis*. To find the optimum time necessary for conjugation to occur on filters, *E. coli* K-12 strain J53 carrying plasmid pJB4JI was mated with *Z. mobilis* strains ZM4 and ZM6, and the frequency of plasmid transfer was measured after increasing lengths of time. It was found that 4 h of incubation gave the highest levels of plasmid transfer; thus, this time was used for all subsequent filter matings.

Longer conjugation times resulted in fewer drug-resistant transconjugants, but also the numbers of *E. coli* K-12 donor cells fell rapidly, with a 10-fold loss of viable cells after 20 h. This may have been due to incubation on a *Zymo-*

monas medium, on which *E. coli* did not grow particularly well, or to some effect caused by incubation in close proximity to *Z. mobilis* cells (such as high levels of alcohol). One of the *Z. mobilis* strains used, strain ZM4, has been reported to have an inhibitory effect on many different bacteria (15, 31), and it is possible that the same inhibition occurs with *E. coli*.

Transfer of R plasmids from *E. coli* and *P. aeruginosa* to *Z. mobilis*. The results in Table 2 show that the IncFII plasmid R1*drd*19 and the IncP1 plasmids pRD1 and pJB4JI can be transferred into *Z. mobilis* by conjugation from *E. coli* hosts. Similarly, the IncP1 plasmid R68.45 can be transferred to *Z. mobilis* from a *P. aeruginosa* host.

All four plasmids were received by *Z. mobilis* at the high frequencies of around 10^{-4} to 10^{-1}. Plasmid R68.45 was transferred most readily, with recipients gaining drug resistances at about a 10^{-1} frequency.

Although resistance to tetracycline was fully expressed in the *Z. mobilis* recipient ZM6 and its derivative ZM61, this was not the case with strains ZM4 and ZM11, where no colonies resistant to tetracycline were obtained above the normal low background level of spontaneous mutants (Table 2).

Evidence for lack of full expression of plasmid markers on initial transfer from *E. coli* to *Z. mobilis* was also obtained in other experiments. For example, when donor strain SB1801(pRD1) was mated with recipient strain ZM11, kanamycin-resistant colonies appeared at a frequency of about 10^{-4}, whereas carbenicillin-resistant colonies were 100 times more frequent (Table 2). However, when all of the well-isolated colonies from both media were replicated onto both kanamycin and carbenicillin media, all colonies tested were resistant to both drugs (Table 3). This phenomenon appeared to be both plasmid specific (occurring only with plasmid pRD1) and strain specific (occurring in ZM4 and ZM11 backgrounds).

Transfer of plasmids between *Z. mobilis* strains. As can be seen from Table 4, when *Z. mobilis* strains carrying any of the four plasmids were used as donors in conjugation experiments with other marked strains of *Z. mobilis*, these plasmids could be transferred at very high frequencies. As would be expected for such plasmids (1, 5, 9, 21), the transfer frequency between *Z. mobilis* strains was considerably higher than for the intergeneric matings described in Table 2.

It was again observed that recovery of tetracycline resistance was strain dependent, with ZM61 being the only background to give full expression of this marker (Table 4). In two experiments using the same donor, strain ZM4(pJB4JI), the ZM61 transconjugants showed es-

TABLE 3. *Expression of drug resistance by Z. mobilis transconjugants*

Conjugation expt	*Z. mobilis* colonies selected for resistance to:	No. of colonies tested	No. of colonies resistant to:		
			Km	Cb	Sp
SB1801(pRD1) × ZM11	Km	130	130	130	
	Cb	118	118	118	
SB1801(pRD1) × ZM4	Km	24	24	24	
	Cb	26	26	26	
SB1801(pRD1) × ZM6	Km	32	32	32	
	Cb	32	32	32	
	Tc	16	16	16	
J53(pJB4JI) × ZM11	Km	171	171		171
	Sp	32	32		32
J53(pJB4JI) × ZM4	Km	24	24		24
ZM4(pJB4JI) × ZM61	Km	87	87		86
ZM4(pJB4JI) × ZM11	Km	478	478		478

TABLE 2. *Transfer of plasmids from E. coli and P. aeruginosa to Z. mobilis*

E. coli K-12 or *P. aeruginosa* donor	*Z. mobilis* recipient	Frequency of marker transfer					
		Km	Cb	Tc	Sp	Cm	Ap
J52(R1*drd*19)	ZM11	4×10^{-2}				5×10^{-2}	5×10^{-2}
	ZM6	1×10^{-2}				2×10^{-1}	1×10^{-3}
J53(pJB4JI)	ZM11	4×10^{-4}			4×10^{-4}		
	ZM4	6×10^{-4}					
	ZM6	9×10^{-3}					
	ZM61	2×10^{-3}			2×10^{-3}		
SB1801(pRD1)	ZM11	1×10^{-4}	1×10^{-2}	1×10^{-8}			
	ZM4	6×10^{-5}	2×10^{-3}	1×10^{-8}			
	ZM6	9×10^{-3}	4×10^{-3}	5×10^{-4}			
	ZM61	7×10^{-3}	5×10^{-3}	6×10^{-1}			
PAO25(R68.45)	ZM11	3×10^{-1}	4×10^{-1}	1×10^{-8}			

TABLE 4. *Retransfer of R plasmids between Z. mobilis strains*

Z. mobilis donor	*Z. mobilis* recipient	Frequency of marker transfer				
		Km	Cb	Tc	Sp	Cm
ZM11(pRD1)	ZM61	8×10^{-1}	9×10^{-3}			
ZM4(pRD1)	ZM61	9×10^{-1}	7×10^{-3}			
ZM6(pRD1)	ZM61	5×10^{-1}	2×10^{-1}	4×10^{-1}		
ZM6(pRD1)	ZM11	1×10^{-1}	4×10^{-1}	8×10^{-7}		
ZM4(pJB4JI)	ZM11	8×10^{-1}			7×10^{-1}	
ZM4(pJB4JI)	ZM61	3×10^{-3}			3×10^{-3}	
ZM11(R68.45)	ZM61	8×10^{-1}	8×10^{-1}	8×10^{-1}		
ZM11(R1*drd*19)	ZM61	8×10^{-1}				7×10^{-1}

sentially the same frequency of inheritance of tetracycline resistance as carbenicillin resistance, whereas very few ZM11 transconjugants were resistant to tetracycline (Table 4).

Also, when strain ZM4 or ZM11, but not ZM6, was a donor of plasmid pRD1 to recipient strain ZM61, fewer carbenicillin-resistant colonies than kanamycin-resistant colonies were observed (Table 4). This lack of expression was somewhat similar to the phenomenon observed on initial transfer of pRD1 to strains ZM4 and ZM11.

Segregation of plasmid markers in *Z. mobilis*. Some segregation of markers from plasmid pRD1 (as distinct from lack of expression) was observed in the background of *Z. mobilis*. This was apparent when this plasmid was transferred from one *Z. mobilis* strain to another by patch matings, with carbenicillin resistance being most frequently lost (Table 5). Segregation of markers occurred in both of the recipient strains tested and did not depend on the original selection medium used to isolate the *Z. mobilis* plasmid-containing donors. No segregation of markers from plasmids R68.45, pJB4JI, and R1*drd*19 was detected (Table 5).

Transfer of *Z. mobilis* chromosomal markers. When one drug-resistant *Z. mobilis* derivative, such as strain ZM11, was used as the donor, with another drug-resistant *Z. mobilis* strain, such as ZM61, as the recipient, it was possible to test for mobilization of chromosomal drug resistances. Although no such transfer could be detected in either patch or filter matings with plasmid pRD1, a low level of transfer of chromosomally encoded rifampin resistance was observed when R68.45 was the mobilizing plasmid used (Table 6). This level of transfer was nearly 100-fold higher than the background level of spontaneous mutation to rifampin resistance. No transfer of streptomycin resistance, the other chromosomal marker in the donor strain, was detected.

DISCUSSION

These results are the first demonstration of R-plasmid gene transfer to and between strains

TABLE 5. *Segregation of plasmid markers in Z. mobilis*

Z. mobilis donor	*Z. mobilis* recipient	No. of donors tested	No. of transconjugant patches resistant to:			
			Km	Cb	Cm	Sp
ZM6(pRD1)	ZM11	40	39	39		
ZM6(pRD1)	ZM61	13	13	9		
ZM11(pRD1)	ZM61	13	13	10		
ZM11(R68.45)	ZM61	14	14	14		
ZM61(R68.45)	ZM11	14	14	14		
ZM11(R1*drd*19)	ZM61	10	10		10	
ZM61(R1*drd*19)	ZM11	10	10		10	
ZM4(pJB4JI)	ZM61	10	10			10

of *Z. mobilis*. Clearly, *Z. mobilis* can act as a recipient or donor for several IncP1 group plasmids and at least one IncFII group plasmid, in a manner similar to many other gram-negative bacteria. Also, these plasmids are transferred, by conjugation on membrane filters, at very high frequencies comparable with frequencies obtained for related bacteria such as *Pseudomonas* and *Rhizobium* (2–5, 16, 18). As with other bacterial species (1, 5, 9, 18, 21), frequencies of plasmid transfer were considerably higher in intraspecies crosses than in intergeneric crosses. However, transfer between homogenic strains of *Z. mobilis* was no higher than between *Z. mobilis* strains of different origins.

Although *Zymomonas* is reported to be fairly closely related to *Rhizobium* and *Agrobacterium*, and also to *Pseudomonas* (6, 25, 32), high-frequency transfer (10^{-1} to 10^{-2}) of the F-type plasmid R1*drd*19 has not been reported for the latter three genera. With various *Rhizobium* species, transfer frequencies of only 10^{-6} or 10^{-7} at most have been reported (1, 9, 10, 30), and attempts to conjugate R1*drd*19 into *P. aeruginosa* by liquid matings have failed (9). It is, however, unlikely that the large differences in frequencies of transfer are simply due to different conjugation methods, since the same low level of transfer of R1*drd*19 to *Rhizobium trifolii* was obtained whether liquid matings or filter matings were used (30; M. Skotnicki and B. Rolfe, unpublished data). Since this plasmid

TABLE 6. *Transfer of chromosomal markers between Z. mobilis strains*

Z. mobilis donor	*Z. mobilis* recipient	Frequency of marker transfer			Frequency of spontaneous mutation	
		Km	Sm	Rp	Sm	Rp
ZM11(R68.45)	ZM61	8×10^{-1}	10^{-8}	9×10^{-7}		
ZM11(pRD1)	ZM61	8×10^{-1}	10^{-8}	10^{-8}		
	ZM61 control				10^{-8}	10^{-8}

can easily be mobilized between various enterobacterial species (9), it may be that *Z. mobilis* is more closely related to organisms such as *E. coli* than previously thought.

In addition to the high-frequency transfer of R1*drd*19 to *Z. mobilis*, it was found that plasmid pJB4JI was stably maintained in *Z. mobilis*. This plasmid was constructed in *E. coli* and is stable in this background (2), but when transferred to several species of *Rhizobium*, it cannot become established (2). This is thought to be due to both restriction and the functioning of at least one Mu phage gene (5, 32). However, the transposon on the plasmid, Tn*5* (20), can become inserted into the *Rhizobium* chromosome and cause mutagenesis (2). An RP4 plasmid carrying Tn*7* and Mu has also been constructed and found to behave in a similar manner when transferred to *Agrobacterium* (32). Plasmid pJB4JI, however, was transferable to *Z. mobilis* at high frequency, conferred resistance to both kanamycin and spectinomycin, and could be retransferred to another *Z. mobilis* strain. Thus, the plasmid appeared to be stable in *Z. mobilis*, as it is in *E. coli*, and would not be useful for mutagenesis by transposition of Tn*5* into the *Z. mobilis* chromosome.

These results obtained with plasmids R1*drd*19 and pJB4JI suggest that *Z. mobilis* may resemble *E. coli* more closely than *Rhizobium* and *Pseudomonas*. This similarity also extends to the deoxyribonucleic acid base composition, with *Z. mobilis* having a guanine-pluscytosine content of 48.5% compared with 50 to 51% for *E. coli*, 59 to 65% for *Rhizobium*, and 58 to 70% for *Pseudomonas* (6).

Evidence was obtained which indicated that mobilization of chromosomal genes can occur between strains of *Z. mobilis* when appropriate transferable plasmids are present in the donor strain, although this needs to be investigated more fully. Together with the fact that various plasmids of different origins can be transferred into and between strains of *Z. mobilis*, this suggests that it should be possible to genetically manipulate this bacterium to use a wider range of carbon sources. Studies are currently under way in this laboratory to introduce genes for the utilization of maltose and starch. If this proves successful, then it is possible that alcohol could be made on a cheaper scale industrially from starch substrates.

ACKNOWLEDGMENTS

The following people are thanked for providing strains: J. Beringer, F. Cannon, N. Datta, J. DeLey, B. Holloway, and V. Skerman. B. Rolfe is thanked for helpful discussions.

This work was supported by a grant from the National Energy Research, Development and Demonstration Council of Australia.

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