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Self-Transmissible Plasmid in *Zymomonas mobilis* Carrying Antibiotic Resistance†

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The cryptic plasmid pRUT41 from *Zymomonas mobilis* was examined for its biological properties. This plasmid was found to be conjugally transferred from *Z. mobilis* CP4 to *Escherichia coli* BM21 and to carry genes for antibiotic resistance (gentamicin, kanamycin, and streptomycin). Covalently closed circular plasmid DNA was isolated from eight transconjugants of *E. coli* BM21. These plasmids were identical in mobility on agarose gels and exhibited the same restriction patterns as the native pRUT41 plasmid isolated from *Z. mobilis*. The plasmid location of the antibiotic resistance genes was further confirmed by transforming *E. coli* BM21 with isolated pRUT41 plasmid from strain CP4 and with plasmids from the transconjugants of BM21. Resistance to streptomycin, kanamycin, and gentamicin was tightly linked and transferred together in all cases.

Zymomonas mobilis is an obligately fermentative, gram-negative bacterium which is used in the tropics for the production of palm wines (18, 25). This organism produces ethanol and carbon dioxide as major fermentation products (9, 10, 25) and has tremendous potential for the commercial production of ethanol. It is reported to be more thermotolerant, more ethanol tolerant, and more osmotolerant than *Saccharomyces* sp. (13, 19, 20), the organism currently used for alcohol production. In addition, *Z. mobilis* is capable of three- to fivefold higher rates of fermentation (2, 11, 13, 14, 16, 17, 20). However, the range of substrates utilized by this organism is restricted to glucose, fructose, and sucrose (25), and this imposes severe limitations on its commercial use.

It may be possible to genetically manipulate this organism to increase its substrate range. Recent studies have shown that plasmids of the P1 and FII incompatibility groups can be conjugally transferred into *Z. mobilis*, and these are relatively stable, expressing antibiotic resistance (6, 22, 23). The mobilization of chromosomal genes from *Z. mobilis* has been reported with plasmid R68.45 (22). However, no suitable vector system has been developed. Different strains of *Z. mobilis* contain a variety of cryptic plasmids ranging in molecular weight from 1.1×10^6 to 46×10^6 (6, 19, 23, 24, 26). All studies agree that strain CP4 (one of the best strains for fermentation [21] contains one large plasmid, which has recently been named pRUT41, and a smaller plasmid (24). No phenotypic trait has yet been assigned to any of the *Z. mobilis* plasmids (23, 24, 26). Recent studies by Stokes et al. (24) have shown homology between many of the plasmids in different strains of *Z. mobilis* by DNA-DNA blot hybridization.

Our strain of *Z. mobilis* was originally obtained from A. Ben-Bassat, Cetus Corporation, Berkeley, Calif. The strain was grown at 30°C with 2% glucose in the medium described by Skotnicki et al. (22). It originally contained two plasmids but lost the smaller plasmid during serial transfers in the laboratory and now contains only the pRUT41 plasmid. In our hands, strain CP4 was resistant to ampicillin (Am) (250 μg/ml), chloramphenicol (Cm) (30 μg/ml), gentamicin (Gm) (100 μg/ml), kanamycin (Km) (500 μg/ml), nalidixic acid (Nx) (500 μg/ml), and streptomycin (Sm) (1,000 μg/ml) in liquid culture and on plates, consistent with previous reports (24, 25). Multiple antibiotic resistance such as that observed in *Z. mobilis* is frequently associated with the presence of R-plasmids in other organisms (1–3, 7, 12, 27). Thus, we have investigated the possibility that the large cryptic plasmid in strain CP4 (pRUT41) may contain genes encoding antibiotic resistance.

To test this possibility, we performed conjugation experiments with four strains of *Escherichia coli*: HB101, 711, BM21, and strain B. Strains BM21 and 711 were obtained from K. B. Sharma, Lady Hardinge Medical College, New Delhi, India, and were sensitive to all of the antibiotics listed except Nx (8). The other strains were obtained from D. H. Duckworth (8). Strain CP4 and the *E. coli* strains were grown to log phase (10^8 cells per ml) at 30°C in unshaken cultures with the medium described for *Z. mobilis* (22). One milliliter of *E. coli* culture was mixed with 3 ml of strain CP4 and incubated without agitation at 30°C for 2 h. This mating mixture was then spread onto MacConkey agar plates (0.1 ml per plate) containing lactose (2 g/liter) and Am (50 μg/ml), Cm (20 μg/ml), Sm (25 μg/ml), Gm (10 μg/ml), or Km (50 μg/ml) and incubated for 48 to 72 h at 30°C. Similar plates were used as mutational controls. Strain CP4 did not grow on lactose MacConkey plates. Conjugations were successful only with strain BM21. Transconjugants were obtained only on Gm, Sm, or Km selection plates and appeared at frequencies of 10^{-4}, 10^{-5}, and 10^{-7} per recipient, respectively. Two types of colonies were obtained, a larger colony, which appeared similar to strain BM21 grown on lactose MacConkey plates lacking antibiotics, and very dark, small, magenta colonies. Approximately 300 colonies were picked (including both types) and screened for antibiotic resistance on plates. All of these transconjugants exhibited resistance to Sm, Gm, and Km. No transconjugant colonies were resistant to Cm or Am. No transconjugants were obtained on plates containing either Am or Cm after mating, indicating a rate of transfer below 10^{-9}. All attempts to transfer antibiotic resistance from strain CP4 to *E. coli* HB101 failed.

To confirm that the pRUT41 plasmid had been transferred from *Z. mobilis* CP4 to *E. coli* BM21, plasmid DNA was isolated from eight of the transconjugants carrying antibiotic resistance markers and from *Z. mobilis* CP4. Plasmids were

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isolated from the *E. coli* strains essentially as described by Clewell and Helinski (4). Plasmids were isolated from strain CP4 by a modification of this procedure in which the lysozyme incubation was carried out at 37°C for 2 h, followed by the addition of pronase (250 μg/ml at 37°C for 1 h). Plasmid DNA was recovered in all of these, and portions of this DNA were digested with restriction enzymes (*Sal*I, *Hin*dIII, and *Eco*RI) for comparison of the restriction fragments by agarose gel electrophoresis. Figure 1 shows a comparison of the restriction fragments obtained from pRUT41 and from plasmid DNA isolated from the transconjugants of BM21. The restriction fragments in the transconjugants were identical to that of the native pRUT41 plasmid in all cases, confirming that this plasmid had been transferred from *Z. mobilis* during conjugation. These restriction patterns agree well with those published for *Eco*RI and *Sal*I by Dally et al. (6). However, these results did not eliminate the possibility that some or all of the antibiotic resistance genes may be chromosomal and mobilized by plasmid pRUT41.

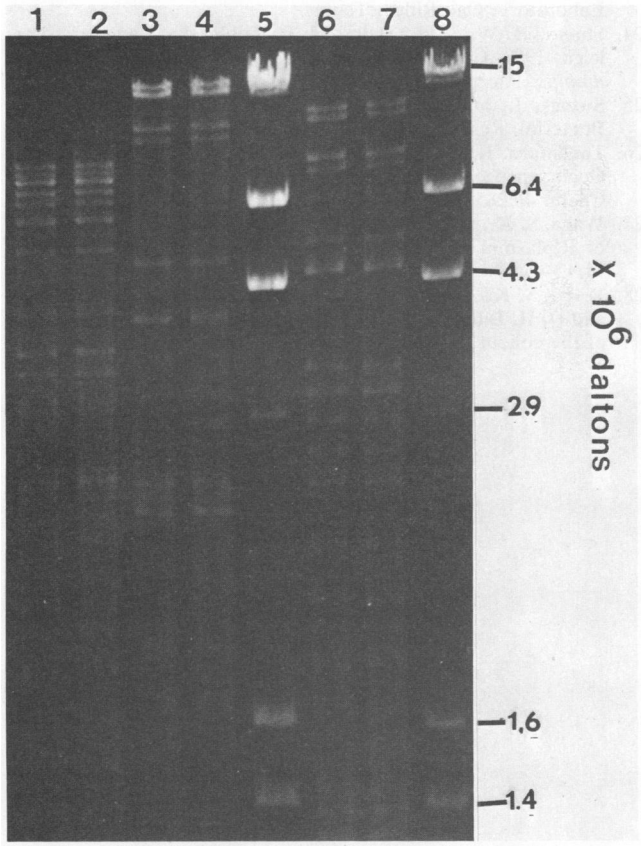

FIG. 1. Comparison of plasmid DNA from *Z. mobilis* CP4 and an antibiotic-resistant transformant of *E. coli* BM21. Purified plasmid DNA was digested with restriction enzymes, and the resulting fragments were purified on 0.8% vertical agarose gels (4 V/cm; 16 h) in Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA [pH 8]). The restriction enzymes were obtained from the Bethesda Research Laboratory, Rockville, Md. and were used according to the recommendations of the manufacturer. The agarose gels were stained with 0.5 μg of ethidium bromide per ml and photographed under UV illumination. Lane 1, *Eco*RI digest of pRUT41; lane 2, *Eco*RI digest of plasmid from transconjugant of strain BM21; lane 3, *Sal*I digest of pRUT41; lane 4, *Sal*I digest of plasmid from transconjugant of strain BM21; lanes 5 and 8, *Hin*dIII digest of phage lambda; lane 6, *Hin*dIII digest of pRUT41; lane 7, *Hin*dIII digest of plasmid from transconjugant of strain BM21.

TABLE 1. Transformation of *E. coli* K-12 strain BM21 with isolated plasmid DNA from *Z. mobilis* CP4[a]

Selection medium	Frequency of transfer	No. of colonies tested	No. of resistant colonies				
			Am	Cm	Gm	Sm	Km
Gm	1×10^4	68	0	0	68	68	68
Sm	2×10^4	103	0	0	103	103	103
Km	1×10^3	60	0	0	60	60	60

[a] Transformation was carried out by the $CaCl_2$ procedure described by Mandel and Higa (15) with 30 ng of DNA per ml containing 10^8 cells. The frequency of transfer was computed as the number of transformants per microgram of DNA.

To establish the plasmid as the location of the three antibiotic resistance genes transferred, purified plasmid DNA from the eight transconjugants and from strain CP4 were used to transform *E. coli* BM21 with selection on lactose MacConkey plates containing one of the five antibiotics. Table 1 summarizes these results with plasmid pRUT41. With all plasmids, resistant transformants were obtained only on Sm, Gm, and Km selection plates. No transformants were obtained on Cm or Am selection plates. Again, small and large colonies were obtained. Both types of colonies were picked and exhibited resistance to the same three antibiotics regardless of the original selection plate. Attempts to transform *E. coli* HB101 were unsuccessful.

Plasmids were isolated from the transformants selected on Km-, Sm-, and Gm-containing plates and the native pRUT41 plasmid from strain CP4. These plasmids exhibited an apparent molecular weight of 50×10^6 on agarose gels and electrophoresed between the pGD32 plasmid (65×10^6 [28]) and the pGC91.14 plasmid (49×10^6 [5]; data not shown). Restriction fragments of the plasmids isolated from the transformants of strain BM21 were identical to that of pRUT41 (data not shown). From these studies, we conclude that the genes encoding resistance to Sm, Gm, and Km in *Z. mobilis* CP4 are carried on the pRUT41 plasmid. The identification of plasmid-borne genes for antibiotic resistance makes this large plasmid a good candidate for the construction of a cloning vector for the genetic improvement of *Z. mobilis*.

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