JOURNAL OF BACTERIOLOGY, July 1992, p. 4500–4503
0021-9193/92/134500-04$02.00/0

Vol. 174, No. 13

Transformation of the Gram-Positive Bacterium *Clavibacter xyli* subsp. *cynodontis* by Electroporation with Plasmids from the IncP Incompatibility Group

MARY C. METZLER,† YONG-PING ZHANG, AND TSEH-AN CHEN*

Department of Plant Pathology, Martin Hall, Cook College, Rutgers University, New Brunswick, New Jersey 08903

Received 31 January 1992/Accepted 22 April 1992

We report the transformation of a gram-positive bacterium, *Clavibacter xyli* subsp. *cynodontis*, with several plasmids in the IncP incompatibility group from gram-negative bacteria. Our results suggest that IncP plasmids may be transferable to other gram-positive organisms. After optimizing electroporation parameters, we obtained a maximum of 2×10^5 transformants per μg of DNA. The availability of a transformation system for this bacteria will facilitate its use in indirectly expressing beneficial traits in plants.

Clavibacter xyli subsp. *cynodontis* is a slowly growing gram-positive coryneform bacterium that grows to high titers in the xylem of Bermuda grass [*Cynodon dactylon* (L.) Pers.] (3, 4, 15). It is closely related to various phytopathogenic *Clavibacter* species but does not cause disease symptoms on Bermuda grass (15). *C. xyli* subsp. *cynodontis* can be transferred to numerous plant species, which has generated much enthusiasm for expressing proteinaceous pesticides such as the crystal toxin from *Bacillus thuringiensis* in the bacterium. The bacterium could thus be used as a vehicle for expressing beneficial traits in commercially important plants without transforming the plant genome (13).

Up to this point, *C. xyli* subsp. *cynodontis* has not been transformed by plasmid DNA, and there is little published information concerning its molecular biology. Our goal is to develop a full set of tools for transforming *C. xyli* subsp. *cynodontis* and for expressing foreign genes in the bacteria with the hope of eventually producing agronomically useful engineered bacteria. We have been working to transform *C. xyli* subsp. *cynodontis* with a plasmid vector by using electroporation, and we report two significant findings in this paper. We show that an IncP family broad-host-range gram-negative plasmid, pLAFR3, can be transferred by electroporation into *C. xyli* subsp. *cynodontis*, and we have defined optimal conditions for transforming *C. xyli* subsp. *cynodontis*, which yielded more than 2×10^5 transformants per μg of DNA.

Isolation and growth of *C. xyli* subsp. *cynodontis*. Samples of Bermuda grass from various locations in the southeastern United States were collected by P. Halisky (Department of Plant Pathology, Rutgers University). Grass leaves from these samples were surface sterilized with 0.1% (wt/vol) $HgCl_2$ for 10 min and washed with sterile H_2O, and then xylem sap was extruded onto DM agar petri dishes by using finger pressure. DM agar is modified from RSD medium (5) and contains (per liter) 17 g of cornmeal agar (Difco Laboratories, Detroit, Mich.), 8.0 g of yeast extract (Difco Laboratories), 1.0 g of $(NH_4)_2HPO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.2 g of KCl, 1.0 g of cysteine (free base), 1.5 ml of 1.0% hemin chloride in 0.5 M NaOH, 10 ml of filter-sterilized 20% bovine serum albumin (fraction V; Sigma, St. Louis, Mo.), and 10 ml of 50% filter-sterilized glucose. Eight different bacterial isolates from plants gathered at different sites were isolated serially as single colonies three times and were stored at −70°C. The only feature differentiating the various isolates was that half contained an approximately 51-kb cryptic plasmid (Table 1).

Transformation of *C. xyli* subsp. *cynodontis*. We found that growth conditions have a very significant effect on the efficiency of transformation. Because the cells grow poorly in liquid culture (with a doubling time of 12 h at best) but quite well on petri dishes (with a doubling time of 4 to 6 h), we always used cells that had been grown as a lawn on DM agar. In addition, we found that cells that were first grown as a single colony from frozen stocks and that were then grown as a lawn gave 50 to 100 times more transformants per μg of pLAFR3 DNA than cells that were grown as a lawn directly from frozen cells. For this reason, *C. xyli* subsp. *cynodontis* was prepared for electroporation by plating onto DM agar plates at a high dilution to give single colonies and was grown at 30°C for 7 days. Single colonies were picked and resuspended in liquid DM medium and then were applied to DM agar plates. These colonies were allowed to grow at 30°C for 5 to 7 days, and then the cells were scraped off of the plates. They were washed twice with sterile 10% glycerol and resuspended to give 5×10^9 cells per ml and then frozen on dry ice-ethanol and stored at −70°C.

For electroporation, 10 μl of thawed cells was mixed with 10 μl of sterile, ice-cold 40% polyethylene glycol 8000 (Fisher Scientific, Pittsburgh, Pa.), and DNA was added in a volume up to 2 μl. The sample was mixed thoroughly and was placed first in the electroporation cuvette and then into the electroporation chamber safe of the BRL Cell-Porator electroporation apparatus (Life Technologies, Bethesda, Md.), and the desired pulse was applied. Initial electrical settings were 8 kΩ, with an internal capacitance of 2 μF, giving a 12-ms pulse at a field strength of 16.6 kV/cm. These are the conditions defined for *Bacillus subtilis* by the manufacturer. After 1 min, the cell suspension was removed from the electroporation chamber and inoculated into 150 μl of liquid DM medium. This was incubated for 4 h at 30°C, and then the cells were plated onto DM agar with the appropriate concentration of antibiotic, as follows: tetracycline, 1.0

* Corresponding author.
† Present address: Division of Plant Physiology, Department of Biology, University of Turku, SF-20500 Turku, Finland.

TABLE 1. Bacterial strains and plasmids used in this study

Strain(s) or plasmid	Description	Source or reference
C. xyli subsp. *cynodontis* isolates		
1, 3, 4, and 7	Do not contain cryptic 51-kb plasmid	P. Halisky
15, 18, 31, and wild type	Contain cryptic 51-kb plasmid	P. Halisky
E. coli DH5α	Standard cloning strain	GIBCO/BRL
pBL2100	*E. coli-Brevibacterium lactofermentum* shuttle plasmid	21
pDM100	*E. coli-Clavibacter michiganense* subsp. *michiganense* shuttle plasmid	17
pRK415	IncP group broad-host-range plasmid	11
pLAFR3	IncP group broad-host-range cosmid	22

μg/ml; kanamycin, 67 μg/ml; neomycin, 25 μg/ml; and gentamicin, 25 μg/ml.

Plasmids that transform *C. xyli* subsp. *cynodontis*. For initial attempts to transform *C. xyli* subsp. *cynodontis*, we chose plasmids that had been developed for transforming other coryneform bacteria (Table 1). Plasmid pBL2100 (21), which carries a gene coding for kanamycin resistance, was used to transform *C. xyli* subsp. *cynodontis*. Kanamycin-resistant colonies became visible after 7 days. To prepare DNA, cells were grown on DM agar containing antibiotic and were resuspended in 100 μl of 10-mg/ml lysozyme (Boehringer Mannheim, Indianapolis, Ind.) to give an A_{600} of 80 to 100 (use of higher concentrations of cells resulted in DNA of poor quality). This suspension was incubated at 37°C for 0.5 h, and then the cells were lysed and the DNA was prepared exactly according to an alkaline-lysis miniprep method used for *Escherichia coli* (18). This DNA was used for restriction digests, agarose gels, and Southern blotting, which were all done by standard methods (18). Because pBL2100 was unstable, we could detect it in *C. xyli* subsp. *cynodontis* only by Southern blotting (data not shown).

We obtained a chimeric plasmid, pDM100, which had been developed from a naturally occurring plasmid from *Clavibacter michiganense* subsp. *michiganense* (17). Several attempts to electroporate *C. xyli* subsp. *cynodontis* by using this plasmid resulted in small antibiotic-resistant colonies. However, these colonies never grew to full size and did not grow on antibiotic when replated. Therefore, it appears that the plasmid was transferred but was not stably maintained.

We then used a broad-host-range plasmid from the IncP family of gram-negative plasmids, pRK415 (11), which contains a tetracycline resistance gene. Tetracycline-resistant colonies were obtained 7 days after electroporation, and the identity of the plasmid was confirmed by Southern blotting. This unexpected result was not seen when the ColE1-based plasmids pBR322 and pBR325 were used. pRK415 was not completely stable, however, as transformants grown on agar with tetracycline formed fewer (about 25% as many) and smaller colonies than those grown without tetracycline.

An IncP plasmid, pLAFR3 (22), was found to readily transform *C. xyli* subsp. *cynodontis*. This plasmid appeared to be moderately stable, being lost at a rate of roughly 1 to 2% per generation. This rate of loss was measured by comparing growth on plates with and without tetracycline and is therefore very approximate. The plasmid was easily detectable in ethidium bromide-stained agarose gels, and the presence of the plasmid in transformed cells was confirmed by Southern blotting. DNA was extracted from three independently obtained transformed colonies from each of seven different transformed isolates. On the basis of the restriction patterns of *Ava*I-digested DNA, the plasmid showed no evidence of rearrangement or deletion for any of the isolates compared with plasmid DNA extracted from *E. coli* (data not shown).

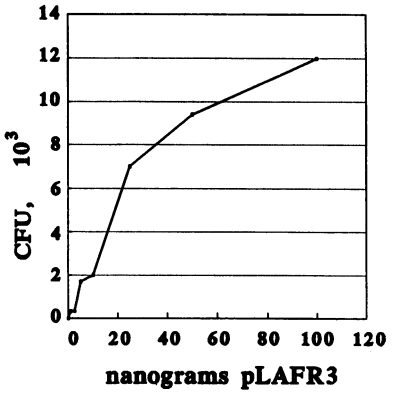

FIG. 1. Effect of DNA concentration on electroporation efficiency under the following conditions: 16 kV/cm, 12-ms pulse, 20% polyethylene glycol, and 1.0×10^8 cells.

The enhanced stability of pLAFR3 in *C. xyli* subsp. *cynodontis* compared with the stability of pRK415 is not readily rationalized, because of both the complexity of the IncP replicon and the fact that various steps of the construction of both precursor plasmids involved loss of undetermined portions of the parent plasmid (6, 7). Although we cannot determine which functions might be responsible for the difference in stability of the two plasmids, it may be possible to engineer an IncP plasmid with enhanced stability in *C. xyli* subsp. *cynodontis*.

Optimization of transformation conditions. The effect of DNA concentration on transformation efficiency can be seen in Fig. 1. The number of transformants increases with DNA concentration but does not appear to be saturated at 100 ng of pLAFR3. In addition, we consistently observed approximately four times more transformants per μg of pRK415 than per μg of pLAFR3. This is probably due to the smaller size of pRK415, an effect seen by other researchers using electroporation to transform bacteria (8).

Optimal electrical conditions were determined by electroporating cells with pLAFR3 at a variety of pulse lengths and field strengths (Table 2). Table 2 shows that lower voltages can be compensated for to some degree by longer pulses. The data demonstrate that optimal conditions are 12 ms at 14 to 16 kV/cm. Freezing and thawing cycles, which increase efficiency of transformation for other gram-positive bacteria (24), resulted in no change in efficiency of transformation (data not shown).

To determine whether restriction barriers might be limiting the ability of DNA derived from *E. coli* to transform *C. xyli* subsp. *cynodontis*, we transformed *C. xyli* subsp. *cynodontis* with pLAFR3 DNA derived from both *E. coli* and *C. xyli* subsp. *cynodontis* (data not shown). There were no differences in transformation efficiency, suggesting that

TABLE 2. Number of transformants for various electrical parameters[a]

Pulse length (ms)	No. (CFU) of transformants at a field strength (kV/cm) of:				
	8	10	12	14	16
6	0	0	0	27	200
12	0	5	40	209	206
24	2	36	0	0	0

[a] Conditions for each parameter: 1×10^8 cells in 20% polyethylene glycol with 1 ng of pLAFR3 DNA.

there are no restriction barriers to transforming *C. xyli* subsp. *cynodontis* with pLAFR3 from *E. coli*.

Our maximum efficiency of transformation with cells grown first as single colonies was 2×10^5 transformants per μg of DNA. This is considerably higher than the maximum efficiency obtained for *C. michiganense* subsp. *michiganense* of 3×10^3 transformants per μg of DNA (17) but is comparable to those seen for other coryneform bacteria transformed by electroporation (in the range of 2×10^4 to 5×10^5 transformants per μg of DNA) (8, 10, 14, 24). No significant difference in the efficiency of transformation was observed for any of the eight isolates tested.

Conclusions. This is the first report of the transfer of a plasmid known to contain only a gram-negative origin of replication into a gram-positive bacterium. Our results are in agreement with numerous recent reports demonstrating that gram-negative and gram-positive organisms can exchange and replicate genetic material from one another. Transfer of DNA by conjugation between gram-negative and gram-positive bacteria has been shown by a number of researchers (2, 16, 19, 23). In addition, there have been previous reports of gram-positive plasmids replicating in gram-negative bacteria (1, 9, 12, 20). Our results, along with the findings of previous researchers, suggest that in nature, gram-negative and gram-positive bacteria may transfer and replicate each other's DNA. More specifically, these results suggest that the IncP family plasmids might be transferable to other gram-positive bacteria, potentially allowing researchers to utilize the large number of different plasmid vectors that have been developed from the IncP plasmid family.

These results represent a significant step forward for studying the molecular biology of *C. xyli* subsp. *cynodontis*, for which no plasmid vectors exist. Although antibiotic selection must be maintained during growth because of the slight instability of pLAFR3, this vector will provide a rapid and convenient method for introducing cloned genes into *C. xyli* subsp. *cynodontis*.

We thank P. Halisky for providing *C. xyli* subsp. *cynodontis*-containing Bermuda grass samples. Plasmids were provided by B. S. Hartley, Imperial College, London; Donald Kobayashi, Department of Plant Pathology, Rutgers University; and Rudolf Eichenlaub, University of Bielefeld, Bielefeld, Germany. We thank Donald Kobayashi and Bradley Hillman for critically reading the manuscript.

This work was supported by a grant from the Center for Interdisciplinary Studies in Turf Science (CISTS), New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick.

REFERENCES

1. **Barany, F., J. Boeke, and A. Tomasz.** 1982. Staphylococcal plasmids that replicate and express erythromycin resistance in both *Streptococcus pneumoniae* and *Escherichia coli*. Proc. Natl. Acad. Sci. USA **79**:2991–2995.
2. **Bertram, J., M. Strätz, and P. Dürre.** 1991. Natural transfer of conjugative transposon Tn*916* between gram-positive and gram-negative bacteria. J. Bacteriol. **173**:443–448.
3. **Chen, T. A., H. J. Su, B. C. Raju, and W. C. Huang.** 1977. A new spiroplasma isolated from Bermuda grass (*Cynodon dactylon* L. Pers). Abstr. Proc. Am. Phytopathol. Soc. **4**:71.
4. **Davis, M. J., A. G. Gillaspie, Jr., R. W. Harris, and R. H. Lawson.** 1980. Ratoon stunting disease of sugarcane: isolation of the causal bacterium. Science **210**:1365–1367.
5. **Davis, M. J., A. G. Gillaspie, Jr., A. K. Vidaver, and R. W. Harris.** 1984. *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cynodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and Bermudagrass stunting disease. Int. J. Syst. Bacteriol. **34**:107–117.
6. **Ditta, G., T. Schmidhauser, E. Yakobson, P. Lu, X.-W. Liang, D. R. Finlay, D. Guiney, and D. R. Helinski.** 1985. Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. Plasmid **13**:149–153.
7. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**:7347–7351.
8. **Dunny, G. M., L. N. Lee, and D. J. LeBlanc.** 1991. Improved electroporation and cloning vector system for gram-positive bacteria. Appl. Environ. Microbiol. **57**:1194–1201.
9. **Goze, A., and S. Ehrlich.** 1980. Replication of plasmids from *Staphylococcus aureus* in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77**:7333–7337.
10. **Haynes, J. A., and M. L. Britz.** 1990. The effect of growth conditions of *Corynebacterium glutamicum* on the transformation frequency obtained by electroporation. J. Gen. Microbiol. **136**:255–263.
11. **Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trollinger.** 1988. Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. Gene **70**:191–197.
12. **Kok, J., J. M. B. M. van der Vossen, and G. Venema.** 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48**:726–731.
13. **Kostka, S. J., S. F. Tomasino, J. T. Turner, and P. W. Reeser.** 1988. Field release of a transformed strain of *Clavibacter xyli* subsp. *cynodontis* (*CXC*) containing a delta-endotoxin gene from *Bacillus thuringiensis* subsp. *kurstaki* (BT). Phytopathology **78**:1540.
14. **Kurusu, Y., M. Kainuma, M. Inui, Y. Satoh, and H. Yukawa.** 1990. Electroporation-transformation system for coryneform bacteria by auxotrophic complementation. Agric. Biol. Chem. **54**:443–447.
15. **Liao, C. H., and T. A. Chen.** 1981. Isolation, culture and pathogenicity to Sudan grass of a corynebacterium associated with ratoon stunting of sugarcane and with Bermuda grass. Phytopathology **71**:1303–1306.
16. **Mazodier, P., R. Petter, and C. Thompson.** 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. **171**:3583–3585.
17. **Meletzus, D., and R. Eichenlaub.** 1991. Transformation of the phytopathogenic bacterium *Clavibacter michiganense* subsp. *michiganense* by electroporation and development of a cloning vector. J. Bacteriol. **173**:184–190.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Schäfer, A., J. Kalinowski, R. Simon, A.-H. Seep-Feldhaus, and A. Pühler.** 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria. J. Bacteriol. **172**:1663–1666.
20. **Serwold-Davis, T. M., N. Groman, and M. Rabin.** 1987. Transformation of *Corynebacterium diphtheriae*, *Corynebacterium ulcerans*, *Corynebacterium glutamicum*, and *Escherichia coli* with the *C. diphtheriae* plasmid pNG2. Proc. Natl. Acad. Sci. USA **84**:4964–4968.

21. **Shaw, P.-C., and B. S. Hartley.** 1988. A host-vector system for an *Arthrobacter* species. J. Gen. Microbiol. **134:**903–911.
22. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169:**5789–5794.
23. **Trieu-Cuot, P., C. Carlier, P. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
24. **Wolf, H., A. Pühler, and E. Neumann.** 1989. Electrotransformation of intact and osmotically sensitive cells of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **30:**283–289.