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Stable Transformation of the Gram-Positive Phytopathogenic Bacterium *Clavibacter michiganensis* subsp. *sepedonicus* with Several Cloning Vectors

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In this paper we describe transformation of *Clavibacter michiganensis* subsp. *sepedonicus*, the potato ring rot bacterium, with plasmid vectors. Three of the plasmids used, pDM100, pDM302, and pDM306, contain the origin of replication from pCM1, a native plasmid of *C. michiganensis* subsp. *michiganensis*. We constructed two new cloning vectors, pHN205 and pHN216, by using the origin of replication of pCM2, another native plasmid of *C. michiganensis* subsp. *michiganensis*. Plasmids pDM302, pHN205, and pHN216 were stably maintained without antibiotic selection in various strains of *C. michiganensis* subsp. *sepedonicus*. We observed that for a single plasmid, different strains of *C. michiganensis* subsp. *sepedonicus* showed significantly different transformation efficiencies. We also found unexplained strain-to-strain differences in stability with various plasmid constructions containing different arrangements of antibiotic resistance genes and origins of replication. We examined the effect of a number of factors on transformation efficiency. The best transformation efficiencies were obtained when *C. michiganensis* subsp. *sepedonicus* cells were grown on DM agar plates, harvested during the early exponential growth phase, and used fresh (without freezing) for electroporation. The maximal transformation efficiency obtained was 4.6×10^4 CFU/μg of pHN216 plasmid DNA. To demonstrate the utility of this transformation system, we cloned a β-1,4-endoglucanase-encoding gene from *C. michiganensis* subsp. *sepedonicus* into pHN216. When this construction, pHN216:C8, was electroporated into competent cells of a cellulase-deficient mutant, it restored cellulase production to almost wild-type levels.

Clavibacter michiganensis subsp. *sepedonicus* (6) (formerly *Corynebacterium sepedonicus* [Spieck. & Kotth.] Skapt. & Burkh.) is a gram-positive coryneform bacterium that causes potato ring rot, an economically important disease in potatoes. This organism causes necrosis of the vascular tissue and thus creates conditions that result in complete destruction of the tuber by secondary infection with soft rot bacteria (15). The use of certified, bacterium-free seed potatoes is one of the most important factors in the control of bacterial ring rot (3, 7). However, the production of certified seed potatoes requires considerable economic resources in North America and Europe. In many potato-growing areas, a zero-tolerance level has been set for this bacterium, and thus strict regulatory measures are used to prevent ring rot infections.

The molecular biology of this very important potato pathogen is poorly understood. The only previous molecular studies of this bacterium have shown that many isolates harbor a 51-kb native plasmid whose function is unknown (5, 19, 20), and they produce extracellular polysaccharides (9, 22), a putative toxic glycopeptide (23–25), and an extracellular cellulase (1, 10).

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We want to investigate the mechanisms of pathogenesis in *C. michiganensis* subsp. *sepedonicus*; however, such an investigation will require efficient molecular tools, such as transformation methods and cloning vectors. As an example, construction of cloning vectors for the tomato pathogen *C. michiganensis* subsp. *michiganensis* allowed workers to identify genes whose products cause wilting (16, 17). Because similar molecular methods have not been available for *C. michiganensis* subsp. *sepedonicus*, we developed a transformation method which allows us to introduce several different plasmids into this bacterium. In this paper we describe the construction of two cloning vectors containing the origin of replication of native plasmid pCM2 of *C. michiganensis* subsp. *michiganensis* and show that these vectors, as well as other *C. michiganensis* subsp. *michiganensis*-derived vectors (16), stably transform *C. michiganensis* subsp. *sepedonicus* and that the transformed bacteria can stably express genes carried on one of these plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The plasmid vectors and strains of *C. michiganensis* subsp. *sepedonicus* and *Escherichia coli* which we used are listed in Table 1. We grew *C. michiganensis* subsp. *sepedonicus* strains at 26°C in our standard rich DM medium (18) with shaking at 250 rpm or on plates containing DM medium and 15 g of corn meal agar (Difco Laboratories, Detroit, Mich.) per liter. Bacteria which contained plasmids were grown on 85-mm agar plates containing the following amounts of antibiotics placed on the surface of the agar and uniformly distributed with a glass rod: 150 and 300 μg of chloramphenicol, 140 and 100 μg of gentamicin, and 400 and 500 μg of neomycin for *C. michiganensis* subsp. *sepedonicus* and *E. coli*, respectively.

Plasmid isolation and recombinant DNA techniques. For large-scale plasmid DNA purification we grew *E. coli* DH5α (Gibco BRL, Gaithersburg, Md.) carrying the desired plasmid with vigorous shaking at 37°C in 2× YT medium (21) and used the Wizard Maxiprep DNA purification system (Promega Corp., Madison,

TABLE 1. Bacterial strains and plasmid vectors used

Strain or plasmid	Description[a]	Source or reference
C. michiganensis subsp. *sepedonicus* strains		
CS2	Strain obtained from diseased potato	R. J. Copeman
CS3-1	Strain obtained from diseased potato	S. H. De Boer
CS4	Strain obtained from diseased potato	S. H. De Boer
CS3-2	Strain obtained from diseased potato	D. Thompson
CS7	Strain obtained from diseased potato	D. Thompson
CS9	Strain obtained from diseased potato	D. Thompson
C. michiganensis subsp. *michiganensis* strains		
NCPPB382	Strain obtained from diseased tomato	17
CMM100	Strain NCPPB382 cured of native plasmids	17
CMM102	CMM100 with plasmid pCM2	17
E. coli DH5α	Standard cloning strain	GIBCO/BRL
Plasmids		
pBR322	Standard cloning vector	4
pCM1	27.5-kb native plasmid from *C. michiganensis* subsp. *michiganensis*	17
pCM2	72-kb native plasmid from *C. michiganensis* subsp. *michiganensis*	17
pDM100	*E. coli-Clavibacter* sp. shuttle vector; Gn^r Nm^r	17
pDM302	*E. coli-Clavibacter* sp. shuttle vector; Cm^r Nm^r	16
pDM302:B1	pDM302 carrying a cellulase gene from *C. michiganensis* subsp. *michiganensis*	16
pDM306	*E. coli-Clavibacter* shuttle vector; Gn^r Nm^r	16
pDM3	pBR322 derivative; Nm^r	17
pDM3313	pDM3 carrying a 16.5-kb fragment containing the origin of replication from pCM2	This study
pHN5	pBR derivative; Gn^r; (flanked by multiple cloning sites) Cm^r Ap^r	This study
pHN5:C8	6.8-kb *Cla*I-*Msc*I fragment from pCS1 in pHN5	This study
pHN205	*E. coli-Clavibacter* shuttle vector; Gn^r Nm^r	This study
pHN216:C8	8.3-kb *Eco*RI-*Hin*dIII fragment of pHN5:C8 in pHN216	This study
pHN216	*E. coli-Clavibacter* shuttle vector; Gn^r Nm^r	This study

[a] Gn^r, gentamicin resistance; Nm^r, neomycin resistance; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance.

Wis.). To isolate small amounts of plasmid DNA, we grew *E. coli* in Terrific Broth (21) with vigorous shaking at 37°C and used a standard alkaline lysis miniprep protocol (21). Plasmid pCM2 was prepared from *C. michiganensis* subsp. *michiganensis* CMM102 as previously described (17). To isolate plasmid DNA from transformed *C. michiganensis* subsp. *sepedonicus*, bacteria were grown for 5 to 7 days on agar plates at 26°C, and the DNA was isolated by a modified alkaline lysis method as described previously for *Clavibacter xyli* subsp. *cynodontis* (13). We used this DNA for restriction analysis and Southern hybridization, which were performed by standard methods (21). Restriction endonucleases were obtained from New England Biolabs, Inc., Beverly, Mass., and were used according to the manufacturer's instructions.

Transformation protocols. We transformed *E. coli* DH5α with plasmid DNA by electroporation by using a Gene Pulser apparatus (Bio-Rad, Richmond, Calif.). We prepared, transformed, and plated *E. coli* DH5α cells as described in the Bio-Rad *Pulse Controller Instruction Manual* (2a). All of the electroporation experiments were done at a capacitance of 25 μF in 0.1-cm cuvettes (Bio-Rad). *C. michiganensis* subsp. *michiganensis* was transformed as described previously (16).

To obtain *C. michiganensis* subsp. *sepedonicus* cells for electroporation, we initially grew bacteria on DM medium plates for 5 days at 26°C to obtain single colonies, since the use of single colonies as initial inoculum for growing competent cells increases the transformation efficiency 50- to 100-fold for the related bacterium *C. xyli* subsp. *cynodontis* (18). Then individual colonies were resuspended in liquid DM medium and either spread as lawns on DM medium plates or inoculated into liquid DM medium.

To obtain growth in liquid media, we grew *C. michiganensis* subsp. *sepedonicus* for 2 days to an optical density at 580 nm of 1.0, after which cells were harvested by centrifugation at 6,000 × *g* for 10 min. We washed the cells twice with sterile 10% (vol/vol) glycerol and resuspended them in the same solution to an optical density at 650 nm of approximately 80. We froze aliquots on dry ice-ethanol and stored them at −70°C or used them directly for electroporation. Alternatively, *C. michiganensis* subsp. *sepedonicus* cells were grown on DM medium plates for 3 or 4 days as a lawn. We collected the cells by scraping them off the plates with a sterile loop. The cells from each plate were resuspended in 1 ml of 10% glycerol and then washed and resuspended as described above.

For electroporation, DNA in a volume up to 2 μl, 20 μl of cells, and 20 μl of ice-cold 40% polyethylene glycol 4000 or 8000 (Sigma Chemical Co., St. Louis, Mo.) were thoroughly mixed in a precooled cuvette on ice. The cuvette was then placed in a pulse chamber, and unless indicated otherwise, the cells were exposed to a single pulse under various electrical conditions. When we applied more than one pulse, the time interval between pulses was 20 s. After applying the desired pulse or pulses, we immediately washed the cells from the cuvette with 1 ml of DM medium or osmotically protective SB medium (per liter: 10 g of tryptone, 5 g of yeast extract, 4 g of NaCl, and 83.7 g of sorbitol, plus $CaCl_2$ and $MgCl_2$ to give final concentrations of 20 mM for each) (28) and incubated them at 26°C for 4 h before we plated them onto DM agar plates containing the appropriate antibiotics (chloramphenicol or neomycin for pDM302 and gentamicin or neomycin for pDM306, pHN205, and pHN216).

Characterization of transformed cells. We measured the transformation efficiency after 4 to 6 days of incubation by calculating the number of CFU per microgram of plasmid DNA used. We tested for the presence of plasmid DNA in putative transformants by Southern hybridization (21). The DNA probes used to detect plasmids in transformed cells were labelled with digoxigenin-11-dUTP, and signals were detected with a chromogenic substrate by using a DIG DNA labelling and detection kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Plasmid stability measurements. We determined the stabilities of cloning vectors in recombinant cells by inoculating 3-ml portions of liquid DM media with single recombinant colonies and growing the organisms in the absence of antibiotic selection at 26°C at 250 rpm on a rotary shaker. After growth for 2 days we inoculated each culture into fresh medium and grew it for an additional 2 days. Reinoculation was repeated once more so that the bacteria were grown without antibiotic selection for a total of 6 days, corresponding to approximately 20 generations. An appropriately diluted aliquot was then plated onto DM agar plates with and without antibiotic selection. We compared the numbers of colonies on the plates in order to assess the stabilities of transformants.

Cloning of a cellulase gene. We isolated a β-1,4-endoglucanase-encoding gene from the native plasmid of *C. michiganensis* subsp. *sepedonicus*, pCS1, by using the cellulase gene from the closely related bacterium *C. michiganensis* subsp. *michiganensis* as a probe. pCS1 was digested with different restriction endonucleases, and the resulting DNA fragments were separated on a 0.5% agarose gel and immobilized on a nylon membrane (21). A 3.2-kb *Eco*RI-*Hin*dIII fragment from pDM302:B1 (16) carrying the cellulase gene from *C. michiganensis* subsp. *michiganensis* was used as a probe in Southern hybridization. A 6.8-kb *Cla*I-*Msc*I fragment was selected from the hybridizing bands for further analysis and was cloned into *Cla*I-*Sma*I sites of pHN5. The resulting plasmid, pHN5:C8, was digested with *Eco*RI and *Hin*dIII, and an 8.3-kb fragment carrying the cellulase gene was cloned into the corresponding sites of pHN216. This construction, pHN216:C8, was electroporated into cellulase-deficient mutant CS7:42 (14). Putative neomycin-resistant and gentamicin-sensitive transformants were characterized to determine their cellulase production and the stability of the cloned gene without antibiotic selection.

RESULTS

Transformation of *C. michiganensis* subsp. *sepedonicus*. Initially, we tested three plasmids (pDM100, pDM302, and pDM306) and six strains (CS2, CS3-1, CS4, CS3-2, CS7, and CS9) (Table 1) of *C. michiganensis* subsp. *sepedonicus* to find the most stable plasmid and most suitable host strain for transformation. In our preliminary attempt to transform intact cells of strain CS2, we used *E. coli-Clavibacter* sp. shuttle vector pDM100, which harbors the origin of replication from native plasmid pCM1 of *C. michiganensis* subsp. *michiganensis* NCPPB382 (17). Competent CS2 cells were prepared from bacteria grown on DM medium plates for 5 to 6 days and used fresh (without freezing) for electroporation. A single pulse with a field strength of 18 kV/cm was used, which gave time constants of 7.9 to 10.8 ms. Under these conditions we obtained 300 transformants per μg of DNA after 7 days of incubation. However, the antibiotic-resistant colonies were small. This suggested that the plasmid was taken up but was not stably maintained, as occurs when the related bacterium *C. michiganensis* subsp. *michiganensis* is transformed with pDM100 (16). To confirm the presence of the plasmid, we blotted *Eco*RV-cut plasmid DNA from the transformed cells and probed with a *Hin*dIII-*Nco*I fragment of pDM306 containing most of the neomycin phosphotransferase gene (16, 17). This gave two well-separated bands for pDM100 (data not shown).

We then used two other plasmids, pDM302 and pDM306, both of which contain the origin of replication of pCM1 and a region required for stable maintenance (16); these plasmids were transformed into all six strains of *C. michiganensis* subsp. *sepedonicus*. We grew bacteria for 6 days on plates, prepared them as described above, and electroporated them at a field strength of 18 kV/cm and a resistance of 600 Ω, which gave time constants ranging from 10.7 to 12.6 ms. The presence of the plasmid was confirmed by probing Southern blots of *Hin*dIII-digested plasmid DNAs from selected transformants with the fragment from the neomycin resistance gene as described previously (data not shown). The number of transformants was about the same for any one plasmid-strain combination no matter what antibiotic was used. However, for both plasmids, the transformation efficiencies varied significantly with different strains of *C. michiganensis* subsp. *sepedonicus*. We tested stabilities as described in Materials and Methods and found strain-to-strain differences; plasmid pDM302 was completely stable in strains CS2, CS4, and CS7 and partially stable in CS3-1, but pDM306 was not stable in any of the strains (Table 2).

Construction of vectors pHN205 and pHN216 and their use for transformation. We decided to construct two new hybrid plasmids carrying the replication region from plasmid pCM2 of *C. michiganensis* subsp. *michiganensis* because the stability of the vectors carrying the pCM1 region sufficient for replication was quite variable in *C. michiganensis* subsp. *sepedonicus*. In addition, the origins of replication for pCM1 and pCM2 are presumably compatible, since these plasmids coexist in the same strain of *C. michiganensis* subsp. *michiganensis*, and it is useful to have vectors derived from different compatible replicons. On the basis of the detailed restriction map, we cloned *Hin*dIII fragments of pCM2 from *C. michiganensis* subsp. *michiganensis* NCPPB382 into *E. coli* by using vector pDM3 (17). When we tested the hybrid plasmids for replication in plasmid-free strain CMM100 (17), we found that the 16.5-kb *Hin*dIII fragment present in plasmid pDM3313 carried the pCM2 replicon (Fig. 1). Introducing deletions into the *Hin*dIII fragment from both ends revealed that an internal 8.9-kb *Ssp*I-*Stu*I fragment was sufficient for replication and stable maintenance in *C. michiganensis* subsp. *michiganensis*. To create *E. coli-Clavibacter* sp. shuttle vector pHN205 (Fig. 2), we joined this fragment to a 6.3-kb DNA fragment carrying the origin of replication of pBR322 (4), the gene for neomycin phosphotransferase of Tn*5* (2), and the gene for gentamicin acetyltransferase of Tn*1696* (26).

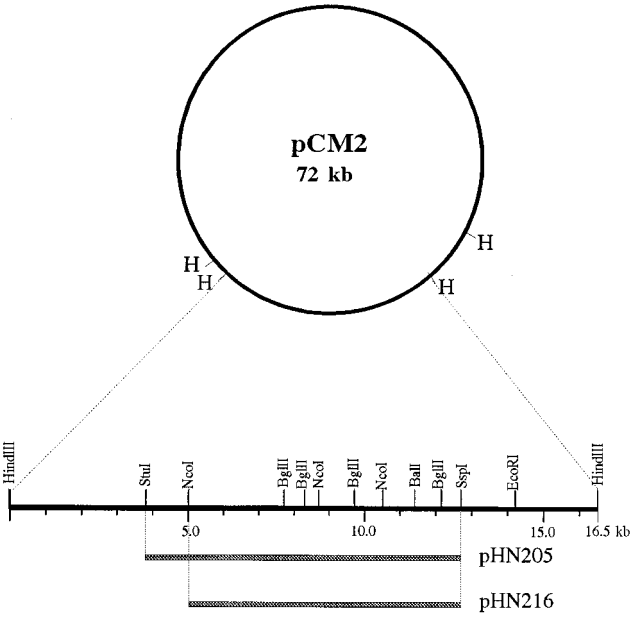

FIG. 1. Physical map of plasmid pCM2 from *C. michiganensis* subsp. *michiganensis* NCPPB382. The *Hin*dIII restriction sites (H) on pCM2 are indicated. The 16.5-kb *Hin*dIII fragment that is present in plasmid pDM3313 and contains the replicon region is shown enlarged. The subfragments of the *Hin*dIII fragment used to construct *E. coli-Clavibacter* sp. shuttle vectors pHN205 and pHN216 are indicated by shaded bars.

In the second vector, pHN216, we shortened the replicon region from plasmid pCM2 to a 7.7-kb *Nco*I-*Ssp*I fragment without any adverse effect on replication and stability in *C. michiganensis* subsp. *michiganensis* and linked it to a 6.1-kb fragment containing the pBR322 origin of replication and the genes for neomycin phosphotransferase and gentamicin acetyltransferase (Fig. 2).

We then transformed *C. michiganensis* subsp. *sepedonicus* with plasmids pHN205 and pHN216 by using electrical parameters similar to those used with the pDM plasmids and again observed strain-to-strain differences in transformation efficiency (Table 2). pHN216 transformed strains CS2 and CS3-1 efficiently, and pHN205 transformed these strains poorly. In contrast, pHN205 transformed CS9 very efficiently, although this strain was barely transformable with pHN216. The stability of transformants varied depending on the combination of plasmids and strains used. Plasmid pHN216 was stably maintained in all strains of *C. michiganensis* subsp. *sepedonicus*, but pHN205 was stable in only two of the six strains tested (Table 2).

Preparation of competent cells. The optimal conditions for preparation of competent *C. michiganensis* subsp. *sepedonicus* cells were determined by varying the growth conditions and pretreatment of cells. Strain CS2 cells were electroporated with 0.5 to 1.0 μg of pHN216 DNA at a field strength of 18 kV/cm with the resistance set to give time constants ranging from 9 to 11 ms. We observed that growing cells as a lawn on agar plates for 3 days resulted in a fivefold increase in transformation efficiency compared with growing cells for 4 days.

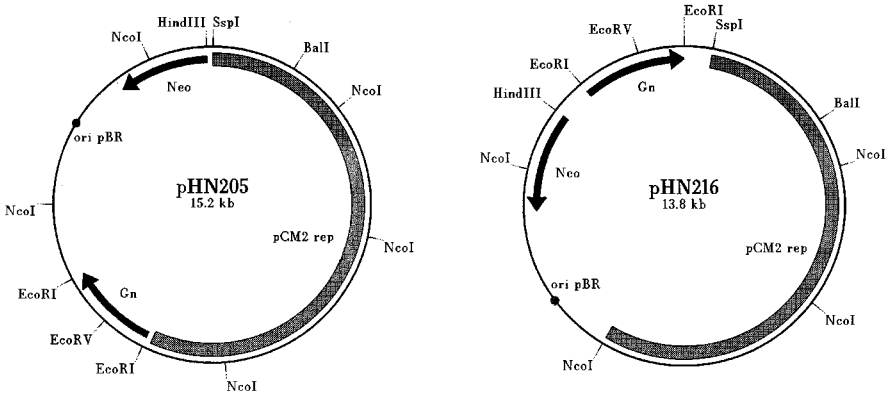

FIG. 2. Physical maps of *E. coli-Clavibacter* sp. shuttle vectors pHN205 and pHN216. Neo, neomycin phosphotransferase; Gn, gentamicin acetyltransferase; ori pBR, origin of replication of vector pBR322; pCM2rep, replicon region of plasmid pCM2.

Furthermore, cells grown as lawns on DM agar plates for 3 days gave approximately eightfold-greater transformation efficiency than cells grown in liquid media to an optical density at 580 nm of 1.0. Competent cells pretreated with lysozyme (40 μg of lysozyme per ml for 20 min at 37°C) immediately after harvesting gave a twofold-higher transformation efficiency than untreated cells grown on plates, but no significant difference was observed for cells grown in liquid media. Prior to electroporation, freezing and thawing of both nontreated and lysozyme-treated competent cells affected the transformation efficiency by reducing the time constants obtained and decreasing the number of CFU per microgram of DNA (data not shown).

Effects of electrical parameters on transformation efficiency. To optimize electrical parameters, we used *C. michiganensis* subsp. *sepedonicus* CS2 cells grown on DM medium for 4 days and frozen and thawed before use. We transformed these cells with plasmid pHN216 DNA at a variety of field strengths and at resistances of 600 and 800 Ω, which gave time constants ranging from 8.8 to 17.7 ms. The best transformation efficiencies were obtained when we treated the cells with two successive pulses with an interval of 20 s by using a field strength of 15 kV/cm and a resistance of 600 Ω, which gave time constants of 9.6 and 10.8 ms. When we used field strengths greater than this optimal field strength, the number of transformants decreased rapidly (Table 3). Using two pulses gave the best efficiencies at lower field strengths (13 to 15 kV/cm), but at 17 kV/cm double pulses gave lower transformation efficiencies and at 19 kV/cm sparking occurred across the sample.

Once we had separately established the optimal growth conditions for competent cells (see above), we combined the optimal electrical parameters with the optimal growth conditions to determine the best transformation efficiency. The maximum transformation efficiency was 4.6×10^4 CFU/μg of pHN216 DNA, which was obtained by using CS2 cells that had been grown on DM agar plates for 3 days, washed twice with 10% glycerol, resuspended to an optical density at 650 nm of 80, and used without freezing. This occurred at a field strength of 15 kV/cm when we used a double pulse and a resistance of 600 Ω, which gave time constants of 11.2 and 10.0 ms.

DNA saturation curve. We grew strain CS2 cells as a lawn on DM medium for 3 days, froze and thawed them before use, and electroporated them with various amounts of pHN216 DNA at a field strength of 18 kV/cm and a resistance of 600 Ω, which gave time constants ranging from 10.9 to 11.6 ms. The relationship between the number of transformants and the amount of DNA was almost linear from 0.125 to 3.0 μg (Fig. 3). A similar observation was made with cells of the gram-positive bacterium *Corynebacterium glutamicum* (27), which supports the hypothesis that the DNA concentration needed for saturation is much greater when electroporation is used than when other transformation procedures are used (11). We did not observe saturation in the range of DNA concentrations that we used; however, the efficiency of transformation decreased rapidly when the amount of DNA was increased from 0.125 to 0.5 μg.

Regeneration of cells after electroporation. To assess the effect of recovery media on transformation efficiency, we electroporated pHN216 plasmid DNA into competent cells of CS2 and allowed them to recover in two different kinds of media, DM medium and SB medium. We used a field strength of 15 kV/cm and a resistance of 600 Ω, which gave time constants of

TABLE 2. Transformation efficiencies of different *C. michiganensis* subsp. *sepedonicus* strains under nonoptimized conditions

Strain	Transformation efficiency (CFU/μg of plasmid DNA), 10^2			
	pDM302	pDM306	pHN205	pHN216
CS2	4.5[a]	1.8	1.0	40.4[a]
CS3-1	0.3	0.2	0.2[a]	34.1[a]
CS4	1.5[a]	0.0	0.1[a]	11.5[a]
CS3-2	0.2	0.5	0.2	0.4[a]
CS7	4.0[a]	3.2	2.8	0.2[a]
CS9	2.0	3.0	31.3	0.8[a]

[a] Plasmid was stably maintained in *C. michiganensis* subsp. *sepedonicus* strain.

TABLE 3. Effect of electrical parameters on transformation efficiency

Resistance (Ω)	Transformation efficiency (10 CFU[2]/μg of DNA) at field strength of[a]:			
	13	15	17	19
600	30.2 (11.2)	41.2 (10.6)	33.9 (10.2)	S[b]
600 + 600[c]	43.9 (10.9, 10.1)	71.4 (10.8, 9.6)	0.8[d] (10.3, 8.8)	S
800	0 (17.7)	23.4 (12.6)	3.2 (11.9)	8.6 (4.1)

[a] Field strength is expressed in kilovolts per centimeter; time constants are indicated in parentheses.
[b] S, sample sparked.
[c] Two pulses were applied with a time interval of 20 s.
[d] Plasmid DNA (0.5 μg) was used while other transformations were done with 1.0 μg of DNA and strain CS2.

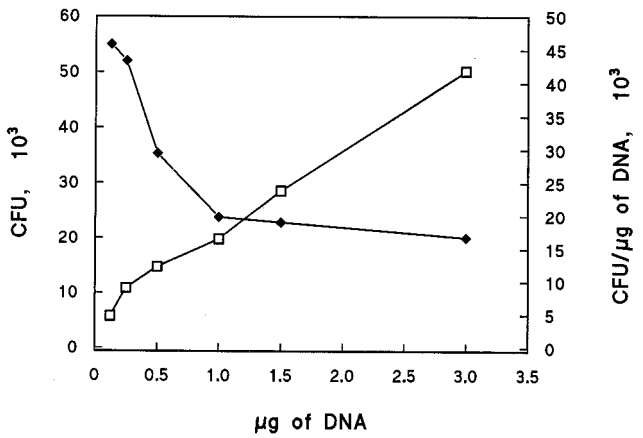

FIG. 3. Effect of DNA concentration on number of transformants per electroporation (□; scale on left) and transformation efficiency (◆; scale on right). *C. michiganensis* subsp. *sepedonicus* CS2 was electroporated with pHN216 plasmid DNA under the following conditions: 18 kV/cm and 600 Ω, giving time constants ranging from 10.9 to 11.6 ms.

10.0 to 11.2 ms. The number of transformants was two to three times higher with osmotically protective SB medium than with DM medium (data not shown). We then examined whether the transformation efficiency was affected by varying the time allowed for the cells to recover after electroporation. Transformed cells were incubated for 1.5, 2.5, or 3.5 h in SB medium at 26°C, and at each timepoint an aliquot was removed and plated onto DM agar containing the antibiotic required for selection. There were no significant differences in the numbers of transformants obtained with the various incubation times.

Use of pHN216 to express cellulase in a cellulase-deficient mutant. To show that the plasmids can be used as cloning vectors for gene expression in *C. michiganensis* subsp. *sepedonicus*, we isolated a cellulase gene from *C. michiganensis* subsp. *sepedonicus* and expressed it by using plasmid pHN216 in a mutant of *C. michiganensis* subsp. *sepedonicus* which did not express the native cellulase. Cellulase-nonexpressing mutant CS7:42 (14) was transformed with pHN216:C8, which contains the cloned native cellulase gene. Four randomly selected neomycin-resistant, gentamycin-sensitive putative transformants all produced cellulase in an indicator plate assay when carboxymethyl cellulose was used as the substrate (Fig. 4). The stability of plasmid pHN216:C8 was tested by growing plasmid-containing bacteria without antibiotic selection in a liquid culture for approximately 20 generations. In mutant CS7:42, the plasmid was completely stable.

DISCUSSION

To develop cloning vectors and transformation methods for *C. michiganensis* subsp. *sepedonicus*, we initially electroporated the bacteria with plasmids containing the origin of replication from pCM1, a native plasmid from *C. michiganensis* subsp. *michiganensis* (16, 17), as such vectors can be introduced into other *Clavibacter* species and subspecies, such as *Clavibacter iranicum*, *C. michiganensis* subsp. *nebraskense*, and *C. michiganensis* subsp. *insidiosum* (17). When we transformed *C. michiganensis* subsp. *sepedonicus* with pDM100, we observed only low levels of transformation, and the plasmid was not stable in the transformants. The stability of pDM100 was also poor in *C. michiganensis* subsp. *michiganensis* (up to 5% segregation per generation), but stability was improved in vectors pDM302 and pDM306 by including a DNA region from pCM1 which is adjacent to the replicon region and which confers stability (16). Thus, the results obtained with pDM302 and pDM306 were more encouraging, and we obtained reasonable but not high levels of transformation. We obtained 450 CFU/μg of DNA for the best combination of strain and vector, which was pDM302 and strain CS2.

To construct improved vectors, we isolated the origin of replication from pCM2, the other native plasmid of *C. michiganensis* subsp. *michiganensis*. We constructed vectors pHN205 and pHN216 in *C. michiganensis* subsp. *michiganensis*, since transformation procedures are better established for this organism than for *C. michiganensis* subsp. *sepedonicus* (16, 17). These vectors also transformed *C. michiganensis* subsp. *sepedonicus*, and the highest efficiency, obtained with strain CS2, was 4.6×10^4 CFU/μg of pHN216 DNA, the efficiency obtained with strain CS2. Although not as high as the values obtained with many gram-negative bacteria, this level of transformation is comparable to the levels obtained for similar gram-positive bacteria (8, 17, 18) and should allow molecular characterization and manipulation of *C. michiganensis* subsp. *sepedonicus.*

For cloning purposes both pHN205 and pHN216 contain a unique *Eco*RV site, which disrupts the function of the gentamicin gene and thus can be used for negative selection of transformants in *E. coli*. Other unique sites that can conveniently be used for cloning are *Ssp*I and *Hin*dIII sites. Insertions cloned into the *Hin*dIII site do not disrupt the function of the neomycin resistance gene. The gentamicin resistance gene, which was found to give rather poor selection in screening transformants of *C. michiganensis* subsp. *sepedonicus*, can be easily removed with *Eco*RI. This provides a unique *Eco*RI cloning site in both plasmids.

We observed significant and unexpected strain-to-strain variation in *C. michiganensis* subsp. *sepedonicus* for the ability to take up plasmid DNA and maintain it stably. This could explain why this important potato pathogen has not been stably transformed previously despite the efforts of workers in several other laboratories, since many plasmid-strain combinations give only transient, low-efficiency transformation. Clearly, it is important to try a number of different plasmid-strain combinations when developing transformation systems for previously untransformed species. We cannot account for the observed

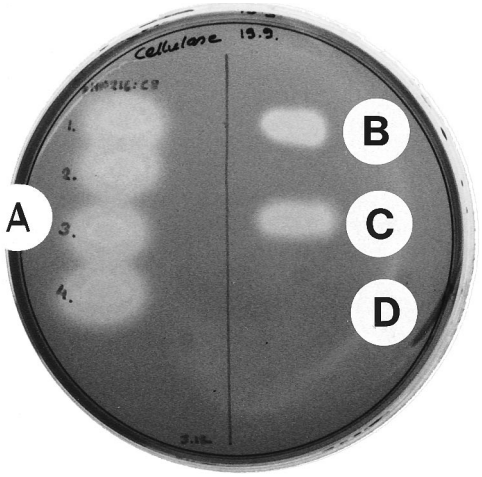

FIG. 4. Expression of cellulase in mutants: cellulase activity on an agar plate containing four CS7:42 transformants carrying pHN216:C8 (A), wild-type strain CS7 (B), a CS7:42 transformant carrying pDM302:B1 (16) (C), and cellulase-deficient mutant CS7:42 (D). Clear halos indicate that cellulase was expressed.

strain-to-strain variations. Maximum transformability does not strictly correlate with stability; for instance, CS9 is the strain that is most efficiently transformed by pHN205, although the plasmid is not stable. However, pHN205 is stable in strain CS3-1, which gives almost 100-fold lower rates of transformation. Low transformation efficiency could be due to restriction systems in the various strains. However, we cannot explain why in different strains the stability of the same plasmid differs.

Within any one strain, different constructions containing the same origin of replication gave plasmids with different stabilities. For example, pDM302 was stable in three of the strains studied, but pDM306 was not stable in any of these organisms, even though the two vectors contain the same pCM1 origin of replication and are almost the same size (16). Similar differences in stability were observed with pHN205 and pHN216, although these plasmids are very similar to each other (Fig. 2). For *C. michiganensis* subsp. *sepedonicus* it seems that the exact size, the orientation of the plasmid components, and/or the antibiotic resistance genes used can affect the stability of a plasmid for reasons that we do not understand.

Several factors affect the efficiency of transformation, and the most important is how the cells are grown. Preparing competent cells from bacteria grown on agar plates instead of in liquid media significantly increased the number of transformants obtained. This may be related to the finding that *C. michiganensis* subsp. *sepedonicus* grows less well in liquid culture than on plates. Our optimal electrical conditions, a field strength of 15 kV/cm with resistance set to give a time constant of 10 to 12 ms, are comparable to those described by other researchers working with similar bacteria (8, 11, 12, 17, 18, 27); we also found that a double pulse gave substantially more transformants at moderate field strengths than a single pulse.

Our goal was to develop a transformation technique which could be efficiently used for cloning genes into *C. michiganensis* subsp. *sepedonicus*. To demonstrate the usefulness of our system for this purpose, we isolated a *C. michiganensis* subsp. *sepedonicus* cellulase gene and expressed it in a mutant strain which did not express cellulase. We found that expression of cellulase was restored to a level near that of the wild-type strain. In addition, although the cellulase-nonexpressing mutant is nonvirulent, we found that restoration of cellulase expression partially restores virulence (14).

In this paper we describe stable transformation of plasmid DNA into *C. michiganensis* subsp. *sepedonicus*. Overall, although pHN216 gave the best results in terms of efficiency and stability, pDM302 is also stable in several strains. Since pDM302 and pHN216 should be compatible, this fact should be useful for genetic manipulation of *C. michiganensis* subsp. *sepedonicus*, as it makes possible construction of transformants containing two different engineered plasmids. Although the efficiency of transformation was highly dependent on various parameters, from preparation of competent cells to electrical conditions, our procedure should be easily adaptable to most laboratories working with *C. michiganensis* subsp. *sepedonicus*. We believe that this transformation system should facilitate investigations of the molecular mechanisms of bacterial ring rot disease.

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