JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 184–190
0021-9193/91/010184-07$02.00/0

Vol. 173, No. 1

Transformation of the Phytopathogenic Bacterium *Clavibacter michiganense* subsp. *michiganense* by Electroporation and Development of a Cloning Vector

DIETMAR MELETZUS AND RUDOLF EICHENLAUB*

Gentechnologie/Mikrobiologie, Fakultät für Biologie, Universität Bielefeld, Postfach 8640, 4800 Bielefeld 1, Federal Republic of Germany

Received 25 June 1990/Accepted 7 October 1990

We constructed a cloning vector for use in the plant pathogenic bacterium *Clavibacter michiganense* subsp. *michiganense*. The vector pDM100 consists of a 3.2-kb restriction fragment of the *Clavibacter* plasmid pCM1 joined to a pBR325 derivative carrying the neomycin phosphotransferase of transposon Tn*5* and the gentamicin acetyltransferase of Tn*1696*. Both antibiotic resistance genes are efficiently expressed in *C. michiganense* subsp. *michiganense*. Although polyethylene glycol-mediated transfection of spheroplasts with the DNA of the *C. michiganense* subsp. *michiganense*-specific bacteriophage CMP1 yielded about 3×10^3 transfectants per μg of DNA, in transformations with plasmid DNA only a very few transformants were obtained. However, the transformation efficiency could be improved by electroporation of intact cells, giving about 2×10^3 transformants per μg of plasmid DNA. Since a transformation procedure and a cloning vector are now available, pathogenicity in *C. michiganense* subsp. *michiganense* can now be analyzed genetically.

Clavibacter michiganense is a gram-positive coryneform bacterium which is a pathogen for a variety of plants of agricultural importance such as tomato, potato, and maize (7, 8). Although strains isolated from different host plants are closely related, the host range is a strain-specific feature, which is reflected in the definition of several subspecies.

Clavibacter michiganense subsp. *michiganense* (Smith) (7), formerly classified as *Corynebacterium michiganense* (Smith) Jensen, causes wilting and bacterial canker of the tomato (*Lycopersicon esculentum*). The possible causal agents, high-molecular-weight glycopeptides, were purified from the culture medium of *C. michiganense* subsp. *michiganense* and partially characterized (14, 15, 20, 21, 27). These glycopeptides cause wilting in an in vitro plant assay, but their role in planta with respect to the development of the disease and their mode of action remain unclear. So far, only the physiological aspects of wilting and canker caused by *C. michiganense* subsp. *michiganense* have been studied. A genetic approach to the problem of pathogenicity was not possible owing to the lack of methods and genetic tools. However, transformation and vector systems have been developed for coryneform bacteria of industrial interest such as *Corynebacterium glutamicum*. Efficient DNA uptake was demonstrated by polyethylene glycol (PEG)-mediated spheroplast transformation (22, 30) and electroporation (11, 28). Alternatively, plasmid RP4-mediated DNA transfer between *Escherichia coli* and a variety of coryneform bacteria was developed recently (23).

In the present report, we describe the development of a shuttle vector system for use in the phytopathogenic bacteria of the genus *Clavibacter*. Although DNA uptake was demonstrated by PEG-mediated spheroplast transformation, satisfying transformation results were only achieved by electroporation of intact *Clavibacter* cells.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *C. michiganense* and *E. coli* and plasmids used in this study are described in Tables 1 and 2. *Clavibacter* bacteriophage CMP1 (9) was obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), Hatching Green, Harpenden, Great Britain.

E. coli strains were grown at 37°C in TBY medium containing 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.2).

Strains harboring plasmids were grown under appropriate selective conditions with 50 μg of neomycin per ml, 10 μg of gentamicin per ml, or 30 μg of chloramphenicol per ml.

Transformation of *E. coli* was done as described by Maniatis et al. (16). Transformants were selected on TBY agar plates with the appropriate addition of antibiotics.

Clavibacter strains were grown at 24 to 26°C in TBY medium supplemented with 5 g of glucose per liter (C medium).

Chemicals. All enzymes and chemicals were obtained commercially. PEG 6000 was purchased from Serva, Feinbiochemica, Heidelberg, Federal Republic of Germany. Zetabind nylon membrane was obtained from Cuno, Inc., Meriden, Conn.

DNA preparation. Plasmid DNA from *E. coli* was prepared for rapid screening by a cleared lysate method. Bacteria from a 5-ml overnight culture were harvested by centrifugation at 3,000 × *g* at 4°C and resuspended in 100 μl of 20% sucrose in 50 mM Tris hydrochloride (pH 8.0). After the bacteria were chilled on ice for 5 min, 10 μl of lysozyme solution (10 mg/ml in H_2O) was added and the mixture was incubated on ice for 10 min. Then 10 μl of 10 mM CDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid) (pH 8.0) was added, and the mixture was kept on ice for an additional 10 min. Subsequently, 100 μl of lysis buffer (10 mM CDTA, 1% Triton X-100, 50 mM Tris hydrochloride, pH 8.0) was added and the lysate was cleared by centrifugation at 15,000 × *g* and 4°C for 30 min. After the addition of 100 μl

TABLE 1. Bacterial strains

Strain	Genotype or description	Resident plasmid(s)	Reference or source
Clavibacter michiganense subsp. *michiganense*			
NCPPB 382	wild type	pCM1, pCM2	NCPPB
CMM100	Curing derivative		This work
CMM101	Curing derivative	pCM1	This work
CMM102	Curing derivative	pCM2	This work
Escherichia coli 294	*endA hsdR pro thi*		10

of H_2O, the supernatant was extracted twice with phenol-chloroform (1:1). Two volumes of ethanol were added to precipitate the DNA, which was then pelleted by centrifugation, dried under vacuum, and finally resuspended in TE buffer (1 mM EDTA, 10 mM Tris hydrochloride, pH 8.0).

Large-scale plasmid DNA preparations were obtained as described by Birnboim and Doly (4). This was followed by further purification on a dye-CsCl gradient.

Depending on plasmid size, large-scale DNA purifications from *C. michiganense* were prepared from a 1-liter culture by either the method of Anderson and McKay (1) or the method of Birnboim and Doly (4); the only modification was that lysozyme treatment (10 mg/ml) was at 37°C for 1 h. Small-scale plasmid DNA was prepared by the following procedure. Cells of a 5-ml overnight culture were pelleted by centrifugation at 3,000 × *g*, washed in 1 ml of TES buffer (50 mM NaCl, 5 mM EDTA, 100 mM Tris hydrochloride, pH 7.5), and pelleted again. The bacteria were resuspended in 100 μl of buffer I (10 mg of lysozyme per ml in 50 mM glucose, 10 mM CDTA, 50 mM Tris, pH 8.0) and incubated for 1 h at 37°C. Immediately after incubation, 200 μl of buffer II (1% sodium dodecyl sulfate [SDS], 0.2 N NaOH) was added and mixed on a vortex mixer. Subsequently, 170 μl of 3 M sodium acetate (pH 4.8) was added, and the mixture was kept on ice for 20 min. Cell debris was removed by centrifugation at 15,000 × *g*, and the supernatant was extracted once with phenol-chloroform. DNA from the supernatant was precipitated by the addition of 2 volumes of ethanol; this was followed by centrifugation at 15,000 × *g*. The DNA pellet was dried under vacuum and resuspended in TE buffer.

Total *Clavibacter* DNA was prepared from 5 ml of a late-log-phase culture as described by Hopwood et al. (13).

The size of DNA molecules was evaluated as described by Southern (25).

Preparation of phage DNA. Phage lysates were prepared from confluent lysis agar plates by the method of Shirako et al. (24) and purified over a CsCl step gradient as described by Maniatis et al. (16). Phage preparations usually had a titer of 1.0×10^{10} PFU/ml. After dialysis against 50 mM NaCl–1 mM $MgSO_4$–10 mM Tris hydrochloride (pH 7.5), the phage suspension was extracted four times with phenol-chloroform (1:1). The phage DNA was dialyzed against TE buffer and stored at 4°C.

Plasmid curing. Plasmids of *C. michiganense* subsp. *michiganense* NCPPB 382 were cured by a modified procedure described by Hendrick et al. (12). C medium (100 ml) in a 500-ml flask was inoculated with 1 ml of late-log-phase culture, which was then grown at 33°C to a titer of about 10^9 cells per ml. Appropriate dilutions were plated on C medium agar plates and incubated at 24°C. Colonies from these plates were tested for the presence of plasmids by colony hybridization with pDM10212 or pDM2313 as the probe.

Colony hybridization. A nylon filter membrane was placed on a petri dish containing solid C medium. The bacterial clones to be tested were transferred to nylon filter membranes and grown for 3 days at 24°C. The filters were then transferred to a stack of Whatman 3MM paper soaked with protoplasting buffer (10 mg of lysozyme per ml in 6.7% sucrose [wt/vol] solution, 1 mM EDTA, 50 mM Tris hydrochloride, pH 8.0) and incubated at 37°C for 1 h. The filters

TABLE 2. Plasmids and phages

Plasmid or phage	Phenotype[a]	Relevant characteristics	Source
pBR322	Ap^r Tc^r	ColE1 replicon	6
pBR325	Ap^r Tc^r Cm^r	ColE1 replicon	5
pDM1	Ap^r Cm^r Neo^r	Derivative of pBR325 carrying NPTII as *Hin*dIII-*Sal*I fragment	This work
pDM2	Ap^r Cm^r Neo^r	pDM1 Δ*Acc*I	This work
PDM3	Ap^r Tc^r Neo^r	NPTII gene inserted in *Pvu*II site of pBR322	This work
pDM10	Gn^r Neo^r	Derivative of pDM2 carrying gentamicin resistance	This work
pDM3212	Ap^r Neo^r	13.5-kb *Cla*I fragment of pCM1 inserted in pDM3	This work
pDM10212	Neo^r	3.0-kb *Bgl*II fragment of pCM1 inserted in pDM10, pCM1-specific probe	This work
pDM2313	Tc^r Neo^r	5.6-kb *Eco*RI fragment of pCM2 inserted in pDM3, pCM2-specific probe	This work
pDM100	Gn^r Neo^r	*E. coli-Clavibacter* shuttle vector	This work
pIB232	Ap^r Gn^r	Promotorless gentamicin resistance flanked by multiple cloning site of pUC19	6a
pSLE80	Ap^r Gn^r		17
pUL62	Ap^r Neo^r	*E. coli-C. glutamicum* shuttle vector	22
CMP1		Lytic, *C. michiganense* subsp. *michiganense*-specific phage	9

[a] Ap^r, Ampicillin resistance; Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Neo^r, neomycin resistance; Gn^r, gentamicin resistance.

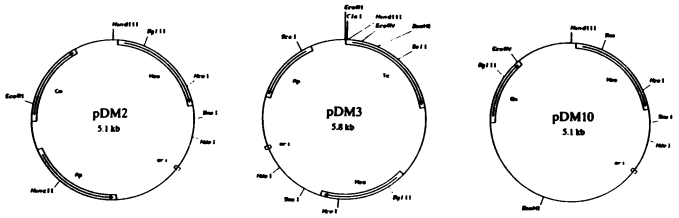

FIG. 1. Physical maps of *E. coli* vectors pDM2, pDM3, and pDM10. Abbreviations: Ap, ampicillin resistance; Cm, chloramphenicol resistance; Gn, gentamicin resistance; Neo, neomycin resistance; Tc, tetracycline resistance; ori, origin of replication of ColE1.

were then carefully placed on a stack of Whatman 3MM paper pretreated with 1 N NaOH–1% SDS for 20 min. After the filters were submerged in 5 M NaCl in 1 M Tris (pH 7.5) for 20 min and in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 min, cell debris was removed from the surface of the filters. The filters were immediately baked for 2 h at 80°C.

For Southern hybridization, DNA was digested with appropriate restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon membranes by blotting with an LKB 2016 Vakugen apparatus.

DNA probes were labeled with digoxigenin-11-dUTP by nick translation as described by Maniatis et al. (16). Hybridization was done at 69°C with 1% blocking reagent, and the results were visualized by using a nonradioactive detection kit from Boehringer GmbH.

Spheroplast transformation and transfection procedure. Spheroplasts of *C. michiganense* subsp. *michiganense* were prepared from 20 ml of late-log-phase culture (10^9 cells per ml) as described by Hopwood et al. (13), using only 2 mg of lysozyme per ml in P buffer. Transformation was done in the presence of 20% PEG 6000 in T buffer (13). For regeneration, spheroplasts were immediately plated onto SB medium by the method of Yoshihama et al. (30). Five hours later, plates were overlaid with 4 ml of SB medium soft agar containing 300 μg of neomycin per ml and incubated at 24°C until colonies appeared. Transfection with phage DNA was performed by the same protocol. However, plaques correlating to transfection events were scored after being overlaid with SB medium containing 0.1 ml of the indicator strain (10^7 cells) and incubating for 3 days at 24°C.

Electroporation. Cells of a 100-ml late-log-phase culture of *C. michiganense* (about 10^9 cells per ml) were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C and washed three times with an equal volume of cold distilled water. Finally, cells were resuspended in 5 ml of distilled water and kept on ice. For electroporation, a Gene Pulser apparatus (Bio-Rad) connected with a pulse controller was used. Cell suspension (100 μl) was mixed with up to 1 μg of DNA in 1 to 2 μl of TE buffer in a precooled 0.2-cm cuvette (Bio-Rad). The cuvette was placed in the pulse chamber, and electroporation was performed under various conditions, i.e., variation of pulse length and field strength. Immediately afterwards, 0.9 ml of C medium was added and the cell suspension was transferred to an Eppendorf tube. For expression of the antibiotic resistance markers, the cells were incubated for 2 h at 24°C and then plated on selective medium. Colonies of recombinant clones usually appeared after 3 days of incubation.

RESULTS

Endogenous plasmids of strain NCPPB 382 and physical mapping of pCM1. *C. michiganense* subsp. *michiganense* NCPPB 382 harbors two large plasmids: pCM1, with a size of 27.5 kb, and pCM2, which has a size of 72 kb. First, it was necessary to construct several derivatives of the *E. coli* vectors pBR322 (6) and pBR325 (5). These were used for the cloning of overlapping DNA fragments of the *Clavibacter* plasmids, allowing the mapping of restriction endonuclease recognition sites and the minimal replicon region. Since detection of transformed cells requires a selective marker and antibiotic resistance genes of *E. coli* cloning vectors are not expressed or are only weakly expressed in coryneform bacteria (18), the neomycin phosphotransferase (NPTII) of Tn*5* and the gentamicin acetyltransferase of Tn*1696*, which are well expressed in a variety of gram-positive bacteria such as *Streptomyces* species (17) or *Corynebacterium* species (22), were integrated into the new *E. coli* vector derivatives. The NPTII gene, obtained as a 1.5-kb *Hin*dIII-*Sal*I fragment from the *E. coli-Corynebacterium* shuttle vector pUL62 (22), was joined with the *Hin*dIII-*Sal*I-digested pBR325, resulting in plasmid pDM1. The internal *Acc*I fragment (pBR322 coordinates 651 to 2246) was deleted after *Acc*I hydrolysis, filling in with Klenow polymerase, and joining of the blunt ends with polynucleotide ligase. This manipulation led to pDM2 (Fig. 1) with a deletion of the *nick/bom* region and a subsequent increase in copy number (26).

Similarly, pDM3 was constructed by inserting the *Hin*dIII-*Sal*I NPTII fragment of pUL62 into the single *Pvu*II site of pBR322 after the ends were filled in with Klenow polymerase and blunt end ligation was done (Fig. 1).

The third vector, pDM10 (Fig. 1), carries the neomycin resistance gene from transposon Tn*5* and the gentamicin resistance gene from Tn*1696*. The construction is illustrated in Fig. 2. Deletion of the *Bgl*II recognition site between the promoter region and the start codon of the NPTII gene (2) did not result in a detectable change of the antibiotic resistance level in *E. coli*.

Subsequently, restriction fragments of pCM1 generated by endonucleases *Bgl*II, *Cla*I, and *Nco*I were inserted into the *E. coli* vector derivatives pDM3 and pDM10. By comparison of the restriction patterns of hybrid plasmids carrying overlapping DNA fragments of pCM1, a physical map of the plasmid was established (Fig. 3).

Plasmid curing. For transformation experiments, the parent strain NCPPB 382, which carries the plasmids pCM1 and pCM2, is not appropriate because incompatibility and recombination can be expected between the resident and the incoming plasmids. Therefore, plasmids were cured by growth of the strain at elevated temperature. Cured derivatives of strain NCPPB 382 were detected by colony hybridization with the labeled hybrids pDM10212 and pDM2313 (Table 2) as probes (data not shown).

The plasmids were cured at a frequency of about 1 to 2%. Three different types of cured derivatives were isolated, the plasmid-free strain CMM100 and the two partially cured

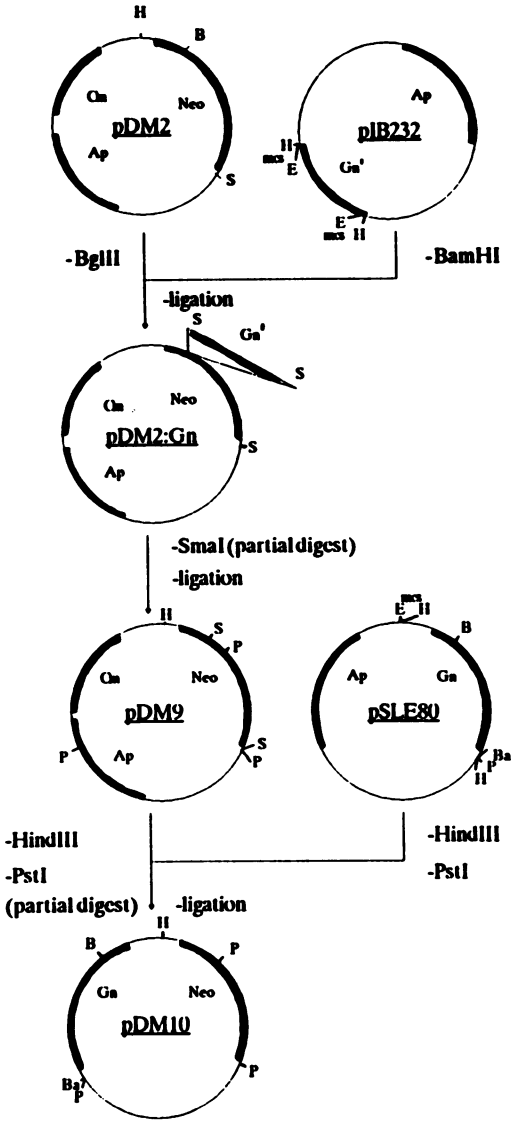

FIG. 2. Construction of pDM10. Abbreviations: Ba, *Bam*HI; B, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sma*I; Ap, ampicillin resistance; Cm, chloramphenicol resistance; Gn, gentamicin resistance; Gn′, promotorless gentamicin resistance gene flanked by multiple cloning site; Neo, neomycin resistance; mcs, multiple cloning site of pUC19 (29).

strains CMM101 with pCM1 and CMM102 with pCM2. The plasmid status of these strains was confirmed either by agarose gel electrophoresis of purified restricted plasmid DNA or by Southern hybridization with total DNA of *C. michiganense*, using a mixture of the labeled plasmids pCM1 and pCM2 as a probe (data not shown).

PEG-mediated transfection and transformation of *Clavibacter* spheroplasts. The initial failures in the transformation of *C. michiganense* with plasmid DNA caused us to test for transfection with the DNA of *Clavibacter* phage CMP1 (9), a linear DNA molecule of about 50 kb. This was successful, and the optimized procedure yielded up to 3×10^3 PFU/μg of DNA (Table 3) when cells were treated for 2 h with 2 mg of lysozyme per ml and 20% PEG 6000. Under these conditions, regeneration of spheroplasts was in the range of 50%.

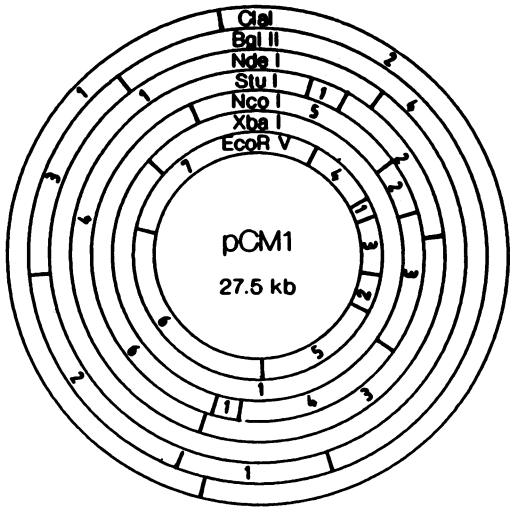

FIG. 3. Restriction endonuclease cleavage map of plasmid pCM1. Restriction fragments are numbered according to their size. The following recognition sites did not occur: *Sca*I, *Pvu*II, *Hin*dIII, and *Eco*RI.

The same protocol was then used for transformation of strain CMM100 with a series of hybrid plasmids consisting of pCM1 restriction fragments inserted into vector pDM3 or pDM10. Transformants were only obtained with the hybrid pDM3212, carrying a 13.5-kb *Cla*I fragment of pCM1 inserted in pDM3 (Fig. 4). However, transformation rates were very low, and generally only 20 transformants per μg of DNA were obtained. As confirmed by the analysis of plasmid DNA from numerous transformed *Clavibacter* clones, the plasmid was always intact (data not shown). Rearrangements or deletions were never observed. Unexpectedly, the transformation efficiency was not improved by using plasmid DNA isolated from *C. michiganense* subsp. *michiganense* CMM100.

Mapping of the region essential for replication in plasmid pCM1. To identify the minimal region required for replication in *C. michiganense*, we constructed several deletion derivatives of pDM3212 and tested them for replication in *C. michiganense* subsp. *michiganense* CMM100. Some of these deletion derivatives are listed in Fig. 4. The *Sca*I-*Xba*I deletion was obtained after digestion and filling in the *Xba*I end with Klenow polymerase followed by ligation. *Bgl*II and *Bam*HI deletions were introduced by partial digestion and religation. In summary, these data indicate that the 3.2-kb

TABLE 3. Effect of duration of lysozyme treatment on phage transfection efficiency and cell regeneration

Duration of lysozyme[a] treatment (h)	Osmotically sensitive cells (%)	Regenerated cells (%)[b]	Transfection efficiency (PFU/μg of DNA)	Transfection frequency[c]
0.5	61.5	96	2.7×10^2	5.4×10^{-7}
1	73	84	6.2×10^2	1.2×10^{-6}
1.5	90	52		
2	92	50	3.4×10^3	6.8×10^{-6}
3	99	19	1.1×10^3	2.2×10^{-6}

[a] Lysozyme concentration was 2 mg/ml in P buffer.
[b] Fraction of regenerated spheroplasts (osmotically sensitive cells).
[c] Transfection per cell; usually 5×10^8 cells were used in a transfection assay.

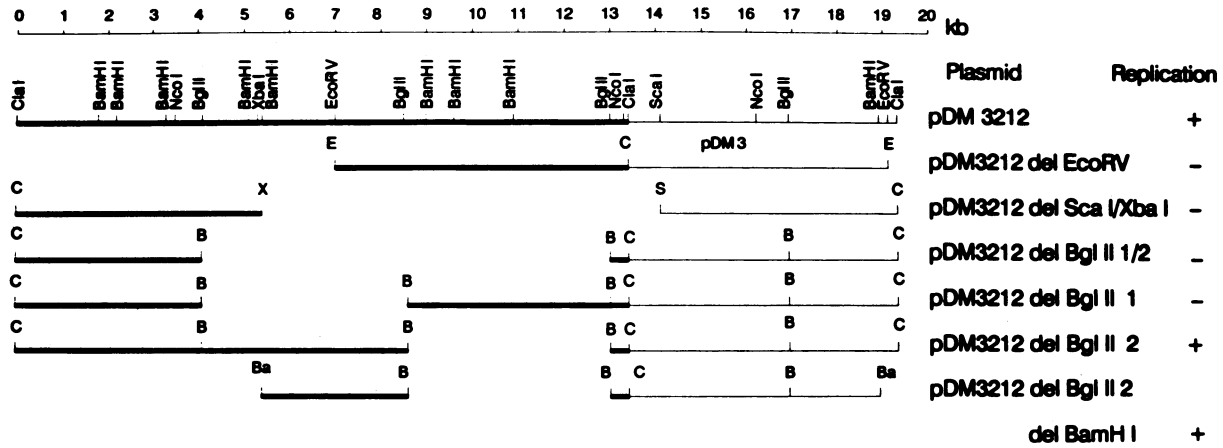

FIG. 4. Mapping of the region of plasmid pCM1 essential for replication in *C. michiganense*. The basic construct pDM3212 linearized at one *Cla*I site is shown at the top of the figure. The thin line represents the vector pDM3; the solid line represents pCM1 DNA. In the deletion derivatives, the solid bars indicate the remaining regions of the 13.5-kb *Cla*I fragment of plasmid pCM1. All derivatives were tested for replication after transformation of *C. michiganense* subsp. *michiganense* CMM100. Abbreviations: Ba, *Bam*HI; B, *Bgl*II; C, *Cla*I; E, *Eco*RV; S, *Sca*I; X, *Xba*I; del, deletion.

*Bam*HI-*Bgl*II fragment is likely to contain all the information required for replication of the hybrid plasmid pDM3212 in *C. michiganense* subsp. *michiganense* CMM100.

Construction of *E. coli-Clavibacter* shuttle vector. The 3.2-kb *Bam*HI-*Bgl*II fragment of pCM1 was inserted into the single *Bam*HI site of pDM10. The resulting shuttle vector, pDM100, had a size of 8.3 kb (Fig. 5) and carried the gentamicin acetyltransferase of transposon Tn*1696* and the neomycin phosphotransferase of Tn*5*, which are both efficiently expressed in *C. michiganense*. Routinely, selection was done with 40 μg of neomycin or gentamicin per ml. We never observed the spontaneous occurrence of resistant clones. When strain CMM100(pDM100) was grown without selective pressure, vector pDM100 segregated at a rate of 1 to 2% per generation. However, this slight instability does not seriously affect the usefulness of this vector in cloning experiments. The copy number of pDM100 in *E. coli* is as high as that described for pBR327. In *C. michiganense* subsp. *michiganense* CMM100, between two and five copies per cell were calculated based on plasmid yields obtained by the standard procedure.

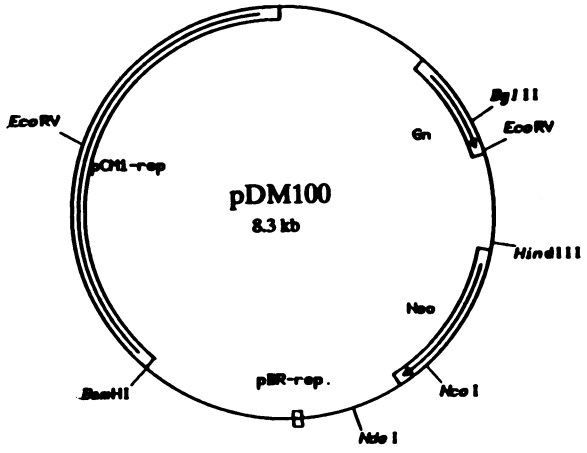

FIG. 5. Physical map of the *E. coli-Clavibacter* shuttle vector pDM100. Abbreviations: pCM1-rep., internal 3.2-kb *Bam*HI-*Bgl*II restriction fragment of pCM1 inserted into the single *Bam*HI site of pDM10; Gn, gentamicin resistance; Neo, neomycin resistance.

Transformation of *C. michiganense* by electroporation. Since the PEG-mediated transformation of *Clavibacter* spheroplasts usually resulted in a very small number of transformed cells, electroporation was tested as a possibility to improve the transformation efficiency. Electroporation was done with 10^9 cells in the presence of up to 0.3 μg of plasmid pDM100 DNA isolated from strain CMM100 or 1 μg of plasmid DNA isolated from *E. coli* (Fig. 6 and Table 4). The mixtures were pulsed throughout the range of pulse duration available as RC constants on the Bio-Rad Gene Pulser. Under optimized conditions (12.5 kV, 13.5 ms) with a 2-h incubation before plating on selective medium, transformation rates were in the range of 2×10^3 transformants per μg of plasmid pDM100 DNA (Fig. 6).

With the linear DNA of phage CMP1, the standard electroporation procedure gave no successfully transfected cells, indicating that DNA uptake in *C. michiganense* during electroporation is more efficient for small, circular DNA molecules. When plasmid DNA isolated from *E. coli* was used, the transformation efficiency dropped by a factor of 100. This is possibly due to an active restriction-modification system in *C. michiganense* subsp. *michiganense* CMM100.

To collect some information on the host range of plasmid vector pDM100, *Clavibacter iranicus* NCPPB 2253, *C. michiganense* subsp. *nebraskense* NCPPB 2581, and *C. michiganense* subsp. *insidiosum* NCPPB 1109 were transformed by electroporation. Although transformation frequencies were lower than those observed with strain CMM100 (data not shown), plasmid pDM100 could be successfully introduced and maintained in these strains. Despite the fact that only three other *Clavibacter* strains were tested, it can be stated that the vector pDM100 can be used for cloning in other phytopathogenic subspecies of *C. michiganense* and related strains.

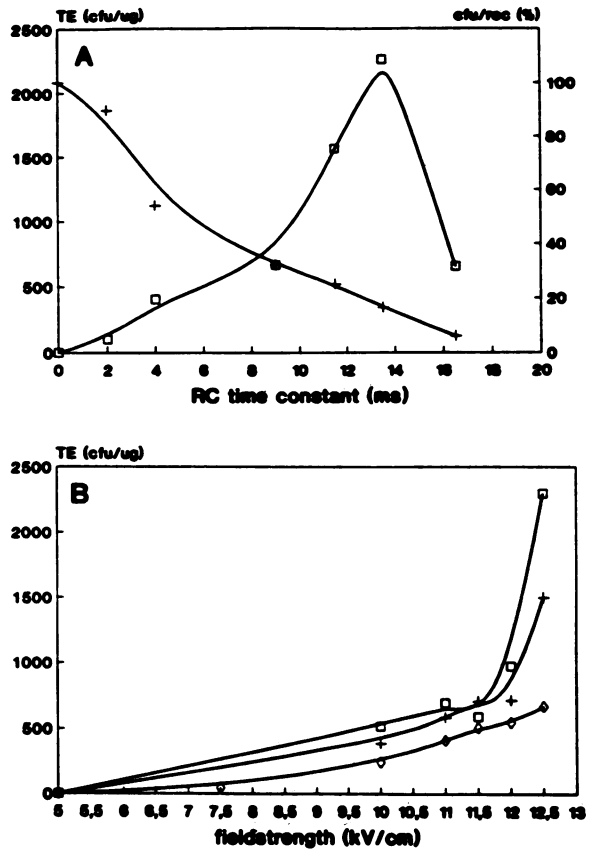

FIG. 6. Parameters affecting the transformation efficiency (TE) by electroporation of *C. michiganense* subsp. *michiganense* CMM100 with pDM100 DNA. Plasmid pDM100 was isolated from strain CMM100. (A) Dependence of the transformation efficiency on pulse length at a field strength of 12.5 kV/cm. Symbols: +, survival of cells expressed as CFU recovered (cfu/rec) in percent; □, transformation efficiency expressed as CFU per microgram of pDM100 DNA. (B) Dependence of the transformation efficiency on field strength at variable RC time constants. Symbols: □, 13.5 ms; +, 11.5 ms; ◇, 9 ms.

DISCUSSION

In this report, we describe the initial stages in the development of recombinant DNA techniques for the phytopathogenic coryneform bacteria of the genus *Clavibacter*.

Plasmid pDM100 is the first vector which can be used in *Clavibacter* species. This vector has the general features of a cloning vector such as unique cloning sites useful for marker inactivation (*Bgl*II, *Nco*I) in addition to several others which may be helpful for further vector modifications (e.g., *Hin*dIII, *Bam*HI).

As pDM100 is an *E. coli-Clavibacter* shuttle vector, this will allow us to link the *E. coli* DNA technology to the *Clavibacter* system. Successful transformation of several other *Clavibacter* species indicates that the use of pDM100 is not restricted to *C. michiganense* subsp. *michiganense*, which extends the possibility of genetic investigation to a variety of phytopathogenic *Clavibacter* strains.

The spheroplast transformation system described in this report is similar to but not as efficient as that reported for other gram-positive bacteria (18, 22, 30). Although transfection gave satisfying results, with up to 3×10^3 transfectants per μg of phage DNA, transformation with plasmid DNA usually yielded only very few transformants. This difference may be due to the fact that in transfection, propagation of the phages occurs in the spheroplasts and does not depend so much on a complete regeneration of a cell wall, as for transformation with plasmid DNA, in which transformants are only detected among regenerated cells. However, since about 50% of the spheroplasts were able to regenerate, there must be other reasons for the low transformation rates with plasmid DNA.

TABLE 4. Effect of pulse length on transformation efficiency, transformation frequency, and cell survival[a]

Time constant (ms)	Transformation efficiency (transformants/μg of DNA)[b]	Transformation frequency (transformants/cell)[c]	% Survival
2.0	1.0×10^2	1.0×10^{-7}	90
4.0	4.0×10^2	4.0×10^{-7}	54
9.0	6.6×10^2	6.6×10^{-7}	32
11.5	1.5×10^3	1.5×10^{-6}	25
13.5	2.3×10^3	2.3×10^{-6}	17
16.6	6.5×10^2	6.5×10^{-7}	6

[a] Plasmid DNA used was pDM100 isolated from *C. michiganense* subsp. *michiganense* CMM100. The field strength was 12.500 V/cm.
[b] Transformants obtained after 2 h of expression followed by plating on C medium containing 40 μg neomycin per ml.
[c] Titer of CMM100 culture prior to electroporation (approximately 10^9 cells per experiment).

Satisfying transformation results were obtained by using electroporation, as has been described for other gram-positive bacteria (3, 19). A drop in the transformation rate when vector DNA isolated from *E. coli* was used indicates the presence of a restriction system in *C. michiganense*. Also, the size and conformation of the DNA seem to affect transformation. Transformation with in vitro-ligated DNA is also possible but requires an extensive dialysis of the DNA versus distilled H_2O prior to use in electroporation.

Finally, we isolated three derivatives of *C. michiganense* subsp. *michiganense* NCPPB 382. These strains differ in their plasmid content and may be helpful in answering questions on the possible involvement of the endogenous plasmids in the pathogenic interaction of *C. michiganense* subsp. *michiganense* with the host plant. Furthermore, since recombinant DNA techniques can now be applied in *Clavibacter* species, we hope to be able to collect more information about the genetic factors determining phytopathogenicity in the genus *Clavibacter*.

ACKNOWLEDGMENTS

This project was supported by the Deutsche Forschungsgemeinschaft (SFB 223/D03).

We thank Inge Broer for plasmid pIB232 and Hendrick Wolf for his introduction to the theory and application of electroporation. We are grateful to Susanne Hemschemeier, Rup Lal, and Andreas Bermpohl for critical reading of the manuscript and to all the members of our laboratory for helpful discussions.

REFERENCES

1. **Anderson, D. G., and L. L. McKay.** 1983. Simple and rapid method for isolating large plasmid DNA from lactic streptococci. Appl. Environ. Microbiol. **46**:549–552.
2. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19**: 327–336.
3. **Belliveau, B. H., and J. T. Trevors.** 1989. Transformation of *Bacillus cereus* vegetative cells by electroporation. Appl. Envi-

ron. Microbiol. **55**:1649–1652.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Bolivar, F.** 1978. Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant molecules. Gene **4**:121–136.
6. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.

6a. **Broer, I.** Unpublished data.

7. **Davis, M. J., A. G. Gillaspie, 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.
8. **Davis, M. J.** 1986. Taxonomy of plant-pathogenic coryneform bacteria. Annu. Rev. Phytopathol. **24**:115–140.
9. **Echandi, E., and M. Sun.** 1973. Isolation and characterization of a bacteriophage for the identification of *Corynebacterium michiganense*. Phytopathology **63**:1398–1401.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
11. **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.
12. **Hendrick, C. A., W. P. Haskins, and A. K. Vidaver.** 1984. Conjugative plasmid in *Corynebacterium flaccumfaciens* subsp. *oorti* that confers resistance to arsenite, arsenate, and antimony(III). Appl. Environ. Microbiol. **48**:56–60.
13. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of Streptomyces: a laboratory manual. John Innes Foundation, Norwich, England.
14. **Krämer, R.** 1986. Preparation and partial characterization of the toxin from *Corynebacterium michiganense* pv. *michiganense* (Smith) Jensen. Zentralbl. Mikrobiol. **141**:327–333.
15. **Krämer, R., and H.-U. Leistner.** 1986. Physiological and cytological aspects of the action of the toxin from *Corynebacterium michiganense* pv. *michiganense* (Smith) Jensen on the host plant. Zentralbl. Mikrobiol. **141**:437–454.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Muth, G., B. Nuβbaumer, W. Wohlleben, and A. Pühler.** 1989. A vector system with temperature-sensitive replication for gene disruption and mutational cloning in *Streptomycetes*. Mol. Gen. Genet. **219**:341–348.
18. **Ozaki, A., R. Katsumata, T. Oka, and A. Furuya.** 1984. Functional expression of genes of *Escherichia coli* in gram-positive *Corynebacterium glutamicum*. Mol. Gen. Genet. **196**:175–178.
19. **Powell, I. B., M. G. Achen, A. J. Hillier, and B. E. Davidson.** 1988. A simple and rapid method for genetic transformation of lactic streptococci by electroporation. Appl. Environ. Microbiol. **54**:655–660.
20. **Rai, P. V., and G. A. Strobel.** 1968. Phytotoxic glycopeptides produced by *Corynebacterium michiganense*. I. Methods of preparation, physical and chemical characterization. Phytopathology **59**:47–52.
21. **Rai, P. V., and G. A. Strobel.** 1968. Phytotoxic glycopeptides produced by *Corynebacterium michiganense*. II. Biological properties. Phytopathology **59**:53–57.
22. **Santamaria, R., J. A. Gil, J. M. Mesas, and J. F. Martin.** 1984. Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*. J. Gen. Microbiol. **130**:2237–2246.
23. **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.
24. **Shirako, Y., A. K. Vidaver, and H.-W. Ackermann.** 1986. Partial characterization of bacteriophages for *Clavibacter michiganense* subsp. *nebraskense*. Ann. Phytopathol. Soc. Jpn. **52**:793–800.
25. **Southern, E.** 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. **68**:153–176.
26. **Twigg, A. J., and D. Sherratt.** 1980. Trans-complementable copy number mutants of plasmid ColE1. Nature (London) **283**:216–218.
27. **Van den Bulk, R. W., H. J. M. Löffler, and J. J. M. Dons.** 1989. Effect of phytotoxic compounds produced by *Clavibacter michiganensis* subsp. *michiganensis* on resistant and susceptible tomato plants. Neth. J. Plant Pathol. **95**:107–117.
28. **Wolf, H., A. Pühler, and E. Neumann.** 1989. Electrotransformation of intact and osmotically sensitive cells of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **30**:283–289.
29. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotides sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
30. **Yoshihama, M., K. Higashiro, E. A. Rao, M. Akedo, W. G. Shanabruch, M. T. Folletie, G. C. Walker, and A. J. Sinskey.** 1985. Cloning vector system for *Corynebacterium glutamicum*. J. Bacteriol. **162**:591–597.