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

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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 Tn5 and the gentamicin acetyltransferase of Tn1696. 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 $3 \times 10^7$ 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 $2 \times 10^3$ 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.

**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. Beetbind 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 x 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 H₂O) 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''-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 x g and 4°C for 30 min. After the addition of 100 µl

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of H₂O, 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 centrifu-
gation, 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 purifica-
tions 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
process. 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 centrifu-
gation 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 × 10¹⁰ PFU/ml. After dialysis against 50 mM NaCl–1 mM
MgSO₄, 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. michi-
ganense 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 10⁹
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 hybridiza-
tion 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 mem-
branes 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 hydro-
chloride, pH 8.0) and incubated at 37°C for 1 h. The filters

<table>
<thead>
<tr>
<th>TABLE 1. Bacterial strains</th>
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<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>Clavibacter michiganense</td>
</tr>
<tr>
<td>subsp. michiganense</td>
</tr>
<tr>
<td>NCPPB 382</td>
</tr>
<tr>
<td>CMM100</td>
</tr>
<tr>
<td>CMM101</td>
</tr>
<tr>
<td>CMM102</td>
</tr>
<tr>
<td>Escherichia coli 294</td>
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<table>
<thead>
<tr>
<th>TABLE 2. Plasmids and phages</th>
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<tbody>
<tr>
<td>Plasmid or phage</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>pBR322</td>
</tr>
<tr>
<td>pBR325</td>
</tr>
<tr>
<td>pDM1</td>
</tr>
<tr>
<td>pDM2</td>
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<tr>
<td>pDM3</td>
</tr>
<tr>
<td>pDM10</td>
</tr>
<tr>
<td>pDM3212</td>
</tr>
<tr>
<td>pDM10212</td>
</tr>
<tr>
<td>pDM2313</td>
</tr>
<tr>
<td>pDM100</td>
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<tr>
<td>pIB232</td>
</tr>
<tr>
<td>pSLE80</td>
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<tr>
<td>pUL62</td>
</tr>
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</table>

* Ap², Ampicillin resistance; Tc², tetracycline resistance; Cm², chloramphenicol resistance; Neo², neomycin resistance; Gt², gentamicin resistance.
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 (10⁶ 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 plasmid 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 (10⁷ cells) and incubating for 3 days at 24°C.

**Electroporation.** Cells of a 100-ml late-log-phase culture of *C. michiganense* (about 10⁷ 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 Tn5 and the gentamicin acetyltransferase of Tn1696, 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 *HindIII-Sall* fragment from the *E. coli-Corynebacterium* shuttle vector pUL62 (22), was joined with the *HindIII-Sall*-digested pBR325, resulting in plasmid pDM1. The internal AccI fragment (pBR322 coordinates 651 to 2246) was deleted after AccI 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 *nckl*om region and a subsequent increase in copy number (26).

Similarly, pDM3 was constructed by inserting the *HindIII-Sall* NPTII fragment of pUL62 into the single *PvuII* 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 Tn5 and the gentamicin resistance gene from Tn1696. The construction is illustrated in Fig. 2. Deletion of the *BglII* 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 *BglII*, *ClaI*, and *NcoI* 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
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 transformation 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 x 10⁸ 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. 2. Construction of pDM10. Abbreviations: Ba, BamHI; B, BglII; E, EcoRI; H, HindIII; P, PstI; S, Smal; Ap, ampicillin resistance; Gm, chloramphenicol resistance; Gn, gentamicin resistance; Gm', promoterless gentamicin resistance; mcs, multiple cloning site of pUC19 (29).](image)

**FIG. 3.** Restriction endonuclease cleavage map of plasmid pCM1. Restriction fragments are numbered according to their size. The following recognition sites did not occur: ScaI, PvuII, HindIII, and EcoRI.

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 ClaI fragment of pCM1 inserted in pDM3 (Fig. 4). However, transformation rates were very low, and generally only 20 transfomers 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 ScaI-XbaI deletion was obtained after digestion and filling in the XbaI end with Klenow polymerase followed by ligation. BglII and BamHI deletions were introduced by partial digestion and religation. In summary, these data indicate that the 3.2-kb

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**TABLE 3.** Effect of duration of lysozyme treatment on plasmid transformation efficiency and cell regeneration

<table>
<thead>
<tr>
<th>Duration of lysozyme treatment (h)</th>
<th>Osmotically sensitive cells (%)</th>
<th>Regenerated cells (%)</th>
<th>Transfection efficiency (PFU/μg of DNA)</th>
<th>Transfection frequency^c^</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>61.5</td>
<td>96</td>
<td>2.7 x 10²</td>
<td>5.4 x 10⁻⁷</td>
</tr>
<tr>
<td>1</td>
<td>73</td>
<td>84</td>
<td>6.2 x 10²</td>
<td>1.2 x 10⁻⁶</td>
</tr>
<tr>
<td>1.5</td>
<td>90</td>
<td>52</td>
<td>3.4 x 10³</td>
<td>6.8 x 10⁻⁶</td>
</tr>
<tr>
<td>3</td>
<td>99</td>
<td>19</td>
<td>1.1 x 10³</td>
<td>2.2 x 10⁻⁶</td>
</tr>
</tbody>
</table>

^a^ Lysozyme concentration was 2 mg/ml in P buffer.

^b^ Fraction of regenerated spheroplasts (osmotically sensitive cells).

^c^ Transfection per cell; usually 5 x 10⁴ cells were used in a transfection assay.
BamHI-BglII 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 BamHI-BglII fragment of pCM1 was inserted into the single BamHI site of pDM10. The resulting shuttle vector, pDM100, had a size of 8.3 kb (Fig. 5) and carried the gentamicin acetyltransferase from transposon Tn1696 and the neomycin phosphotransferase of Tn5, 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.

**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 10⁸ 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 × 10⁴ transformants per μg of plasmid pDM100 DNA (Fig. 6).

With the linear DNA of phage CM1, 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.
TABLE 4. Effect of pulse length on transformation efficiency, transformation frequency, and cell survival*

<table>
<thead>
<tr>
<th>Time constant (ms)</th>
<th>Transformation efficiency (transformants/µg of DNA)</th>
<th>Transformation frequency (transformants/cell)</th>
<th>% Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>$1.0 \times 10^2$</td>
<td>$1.0 \times 10^{-7}$</td>
<td>90</td>
</tr>
<tr>
<td>4.0</td>
<td>$4.0 \times 10^2$</td>
<td>$4.0 \times 10^{-7}$</td>
<td>54</td>
</tr>
<tr>
<td>9.0</td>
<td>$6.6 \times 10^2$</td>
<td>$6.6 \times 10^{-7}$</td>
<td>32</td>
</tr>
<tr>
<td>11.5</td>
<td>$1.5 \times 10^3$</td>
<td>$1.5 \times 10^{-6}$</td>
<td>25</td>
</tr>
<tr>
<td>13.5</td>
<td>$2.3 \times 10^3$</td>
<td>$2.3 \times 10^{-6}$</td>
<td>17</td>
</tr>
<tr>
<td>16.6</td>
<td>$6.5 \times 10^2$</td>
<td>$6.5 \times 10^{-7}$</td>
<td>6</td>
</tr>
</tbody>
</table>

* Plasmid DNA was used pDM100 isolated from C. michiganense subsp. michiganense CMM100. The field strength was 12,500 V/cm.
* Transformants obtained after 2 h of expression followed by plating on C medium containing 40 µg neomycin per ml.
* Titers of CMM100 culture prior to electroporation (approximately $10^9$ cells per experiment).

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 (BglII, NcoI) in addition to several others which may be helpful for further vector modifications (e.g., HindIII, BamHI).

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 $3 \times 10^3$ 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.

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 H2O 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.

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