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Evidence for Plasmid-Encoded Virulence Factors in the Phytopathogenic Bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382

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The tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382, which causes bacterial wilt, harbors two plasmids pCM1 (27.5 kb) and pCM2 (72 kb). After curing of the plasmids, bacterial derivatives were still proficient in the ability to colonize the host plant and in the production of exopolysaccharides but exhibited a reduced virulence. When one of the two plasmids is lost, there is a significant delay in the development of wilting symptoms after infection and a plasmid-free derivative is not able to induce disease symptoms. By cloning of restriction fragments of both plasmids in the plasmid-free strain CMM100, two DNA fragments which restored the virulent phenotype were identified. Further analysis suggested that a fragment of plasmid pCM1 encodes an endocellulase which is involved in the expression of the pathogenic phenotype.

Clavibacter michiganensis subsp. *michiganensis* is a pathogen of the tomato (*Lycopersicon esculentum*) that causes bacterial wilt and canker (8, 22). The bacteria infect the host plant via wounds, invade the xylem, and cause a systemic infection. To explain the mechanisms for the induction of bacterial wilt several hypotheses have been presented. Because purified exopolysaccharides (EPS) produced by the bacteria caused wilting in an in vitro assay with tomato seedlings (19, 20, 25, 26), EPS were inferred to abolish water transport by plugging the xylem vessels. It was also shown that EPS inhibit callus development from tomato protoplasts (28). Furthermore, it was suggested that wilting may be caused by bacterial exoenzymes attacking xylem vessels and adjacent parenchymatic cells (4, 31). However, no clear evidence as to how and to what extent these different phenomena contribute to wilt induction in the host plant exists.

As a first step to eventually understanding the particular mechanisms of wilt induction, we have developed a vector and transformation system for *C. michiganensis* subsp. *michiganensis* (16). In the present report, we describe that plasmids pCM1 and pCM2 (16) of the pathogenic strain NCPPB382 are involved in the development of disease symptoms. By using improved *Clavibacter* vectors, DNA fragments of both plasmids were cloned in order to identify pathogenicity genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Growth conditions were chosen as described previously (16). For EPS isolation, bacteria were grown in yeast extract medium (24). For reisolation of bacteria from infected plants, a modified SCM medium (10) with 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KH_2PO_4, 10 g of sucrose, 15 g of boric acid, 0.1 g of yeast extract, 30 mg of nalidixic acid, and 15 g of agar per liter was used. *Clavibacter* strains harboring plasmids were grown on selective LB medium (16) containing, per milliliter, 50 μg of neomycin, 10 μg of chloramphenicol, or 40 μg of gentamicin.

Recombinant DNA techniques. The preparation of plasmid DNA from *Escherichia coli* and *C. michiganensis* subsp. *michiganensis* was previously described (16). *E. coli* was transformed according to the method of Maniatis et al. (15), and the electroporation of *Clavibacter* strains was carried out as described previously (16). For Southern hybridization, DNA was digested with appropriate restriction enzymes, separated by agarose gel electrophoresis, and transferred to nylon membranes (Hybond-N; Amersham Buchler, Braunschweig, Germany) 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. (15). Hybridization was done at 68°C with 1% blocking reagent and visualized by using a nonradioactive detection kit from Boehringer GmbH, Mannheim, Germany.

Assay for exoenzyme activities. Endocellulase activity was detected on M9CMC agar plates consisting of M9 medium (15) lacking glucose but supplemented with 0.1% (wt/vol) yeast extract and 0.5% (wt/vol) carboxymethylcellulose. Clones to be tested were incubated for 4 to 5 days at 24 to 26°C. Plates were stained with 0.1% (wt/vol) Congo red for 20 min and finally bleached with 1 M NaCl (1). Endocellulase activity is detected as a clear, yellowish halo around producing colonies. Plate assays for xylanase and polygalacturonase activity were done according to the methods of Ghangas et al. (11) and Collmer et al. (6, 7), respectively.

Virulence assay. The standard infection procedure was done according to the method of Van Steekelenburg (29). Four-week-old tomato plants (*Lycopersicon esculentum* cv. Moneymaker) were infected by cutting off the petiole of the first true leaf near the stem with a scalpel which had been dipped into the bacterial suspension (10^9 CFU/ml) to be tested. By this method, usually 100% of the plants were successfully infected. Plants were then transferred to a growth chamber and incubated at 25°C for 16 h (12,000 lx; Fluora-77 lamps; Osram) and 19°C for 8 h (night) with a relative humidity of 80%.

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TABLE 1. Bacterial strains and plasmids

Organism or plasmid	Genotype, description, or phenotype	Resident plasmid or relevant characteristic	Reference or source
Organism			
C. michiganensis subsp. *michiganensis*			
NCPPB382	Wild type	pCM1, pCM2	National Collection of Plant Pathogenic Bacteria
CMM100	Cured derivative		16
CMM101	Cured derivative	pCM1	16
CMM102	Cured derivative	pCM2	16
NCPPB3123	Wild type		National Collection of Plant Pathogenic Bacteria
E. coli Ec294	*endA hsdR pro thi*		13
Plasmid			
pDM3	Ap^r Tc^r Neo^r		16
pDM10	Gn^r Neo^r		16
pDM100	Gn^r Neo^r	*E. coli-Clavibacter* sp. shuttle vector	16
pDM302	Cm^r Neo^r	*E. coli-Clavibacter* sp. shuttle vector	This work
pDM302:B1	Neo^r	3.2-kb *Bgl*II fragment of pCM1 in pDM302	This work
pDM3212	Ap^r Neo^r	13.5-kb *Cla*I fragment of pCM1 in pDM3	16
pDM3212 Δ *Bgl*II-2	Ap^r Neo^r		16
pDM3212 Δ *Bgl*II-2 Δ*Bam*HI-5	Ap^r Neo^r		16
pJD2107	Neo^r	3.75-kb *Bgl*II fragment of pCM2 in pDM306	This work
pSVB30	Ap^r		2

One experimental group generally consisted of 30 plants. The virulent phenotype of *Clavibacter* strains was estimated by examining infected plants for wilting symptoms and determining the plant biomass. Over a period of up to 42 days, plants were examined every day for the development of wilt symptoms, i.e., the occurrence of leaf curling. A virulent phenotype will eventually cause wilting in all infected plants. A more quantitative estimation of virulence is possible by determining the wilting index, defined as the number of days required until 50% of 30 plants showed wilting symptoms.

For a comparison of plant biomass, the dry weights of infected and noninfected plants were determined. Five weeks after infection, 10 plants were cut 1 cm above the ground and dried for 3 days at 110°C. The dry weight of infected plants was expressed as a percentage of the noninfected control plants (100%). All experiments were repeated several times.

Determination of bacterial titers in planta. At various times after infection, five tomato plants were harvested by being cut 1 cm above the ground and homogenized in 10 ml of 68.5 mM NaCl in 0.02 M phosphate buffer (pH 7.2) by being ground in a mortar. Serial dilutions of the homogenate were plated on modified SCM medium semiselective for *Clavibacter* organisms (10). Colonies were scored after incubation at 24 to 26°C for 5 days.

Isolation of EPS. Cultures (50 ml) were grown in a yeast extract medium (24) at 25°C for 12 days (to the late stationary phase) on a rotary shaker. Cells were removed by centrifugation at 16,000 rpm (25,000 × *g*) for 30 min. The supernatant was first passed through a cellulose-acetate filter (pore size, 0.45 μm) to remove the remaining bacterial cells (steril filtration) and then passed through an Amicon XM50 filter to remove components with molecular masses of 50 kDa or lower. The retained material was washed and finally adjusted to 5 ml with distilled water. The EPS concentration was determined by the anthrone method against a glucose standard (9).

RESULTS

Correlation between plasmids and virulence. The pathogenic strain *C. michiganensis* subsp. *michiganensis* NCPPB382 harbors two plasmids, pCM1 (27.5 kb) and pCM2 (72 kb) (16) (Fig. 1). Plasmid curing should therefore contribute to answering the question whether these two plasmids are involved in pathogenicity. The isolation of cured derivatives of strain NCPPB382, CMM100 (plasmid free), CMM101 with pCM1, and CMM102 with pCM2 has been described previously (16). The plasmid status of these strains was confirmed by Southern hybridization, as shown in Fig. 2. In the plasmid-free strain CMM100 (Fig. 2, lane 4), no bands which are typical for plasmids pCM1 and pCM2 were present, but there were positive hybridization signals in the high-molecular-weight range. The fact that we were not able by any means to detect a plasmid in CMM100 indicates that there are some homologies between chromosomal DNA and plasmids pCM1 and pCM2.

These cured derivatives were tested by infecting tomato plants and scoring them for the development of wilting symptoms. Wilting was observed for the parental strain NCPPB382, CMM101 (pCM1), and CMM102 (pCM2). However, in infections with the last two, it took generally 4 to 6 days longer for 50% of the plants to exhibit wilting symptoms (Table 2). It was surprising to observe that infections with the plasmid-free strain CMM100 failed to induce any wilting symptoms; even 6 weeks after infection, plants looked completely healthy.

Corresponding results were obtained when the biomass of infected tomato plants was determined (Table 2). Because of extensive wilting, the biomass of plants infected with NCPPB382 was reduced to 35% of that of noninfected control plants. The greater relative biomass after infection by CMM101 and CMM102 is a consequence of retarded wilting. Although CMM100 can be considered apathogenic since the strain did not induce any wilting symptoms in the host plant, a slight reduction of plant biomass to 75% of that

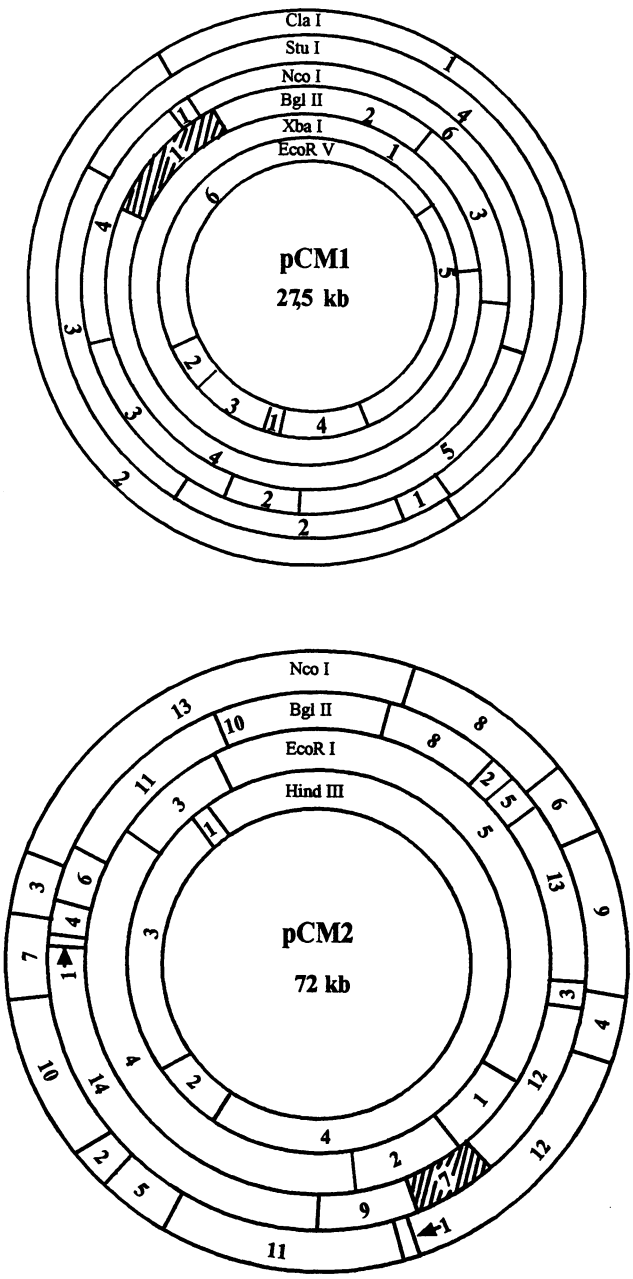

FIG. 1. Physical maps of plasmids pCM1 and pCM2 obtained by analysis of restriction fragments generated by enzymes as indicated. Restriction fragments are numbered according to ascending molecular weights. Fragments *Bgl*II-1 of pCM1 and *Bgl*II-7 of pCM2 (hatched sections) carry determinants for pathogenicity and were cloned in pDM302:B1 and pJD2107, respectively.

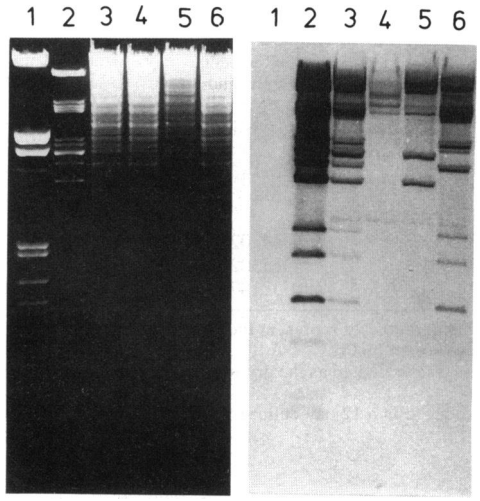

FIG. 2. Analysis of the plasmid status of cured derivatives by Southern hybridization. Total DNA was digested with *Bgl*II and hybridized against a probe consisting of a mixture of digoxigenin-11-UTP-labeled pCM1 and pCM2 plasmid DNA. Lanes: 1, phage λ DNA digested with *Eco*RI-*Hin*dIII; 2, mixture of purified plasmids pCM1 and pCM2; 3, NCPPB382; 4, CMM100; 5, CMM101; 6, CMM102.

of noninfected control plants was still observed. These results suggest that plasmids pCM1 and pCM2 contribute to the pathogenic phenotype of strain NCPPB382, i.e., induction of wilting in infected tomato plants.

EPS production. *C. michiganensis* subsp. *michiganensis* produces high-molecular-weight EPS which can induce wilting of tomato cuttings in an in vitro assay (19, 20, 27). In order to determine whether plasmids affect EPS synthesis, the amounts of EPS produced by NCPPB382 and the cured derivatives were compared. As shown in Table 2, no significant difference in EPS production was observed. Furthermore, in bioassays in which the phytoaggressive activity of EPS was tested with tomato cuttings according to the procedure described by Van Den Bulk et al. (27), EPS preparations of all strains had the same activity (data not shown). This shows that EPS production in culture is not affected by the plasmid status of the strains. Furthermore, a biochemical analysis of the EPS produced by the different strains showed that the chemical compositions were identical (4a).

Colonization of the host plant. Since it is possible that differences in virulence result from variations in the ability to colonize the host plant, the bacterial titers in tomato plants infected with strain NCPPB382 and its derivatives were determined at various times after infection with a semiselective medium for *C. michiganensis* which suppresses growth of saprophytic bacteria and fungi (10). As a control we included strain NCPPB3123 in the experiment, a strain which is apathogenic and has lost the ability to effectively colonize tomato plants. We found no indication that our titer estimations were affected by saprophytic growth of *C. michiganensis* subsp. *michiganensis* on the plant surface, particularly since the titers determined in the xylem fluid were in the same range. Therefore, we omitted the surface sterilization of the plants prior to titer determinations.

Although NCPPB382 and its derivatives show slight differences in the increase of the titer during the first 3 days, all strains, regardless of their plasmid status, reached a final bacterial titer of about 10^9 CFU/g of plant tissue after 9 days (Fig. 3), and this titer was maintained during later stages of infection.

It is surprising that CMM100, despite its proficient endophytic growth and EPS production, does not induce any disease symptoms, i.e., wilting. Only a slight growth retardation in the infected plant as indicated by the reduced biomass was observed (Table 2).

Test for exoenzymes. Since it was proposed that bacterial

TABLE 2. Phytopathogenic properties of strain NCPPB382, its cured derivatives, and CMM100 carrying hybrid plasmids

Strain	Resident plasmid	Wilt induction[a]	Biomass (%)[b]	EPS (g/cell)[c]	Endocellulase activity
NCPPB382	pCM1, pCM2	+ (12)	35 ± 14	2.0×10^{-13}	+
CMM100			75 ± 27	2.1×10^{-13}	−
CMM101	pCM1	+ (18)	62 ± 22	2.5×10^{-13}	+
CMM102	pCM2	+ (16)	50 ± 23	2.9×10^{-13}	−
CMM100	pDM302		76 ± 25	NT	−
CMM100	pDM302:B1	+ (18)	63 ± 19	NT	+
CMM100	pJD2107	+ (17)	56 ± 10	NT	−

[a] Values in parentheses represent the wilting index, the number of days after infection when 50% of 30 infected plants showed wilting symptoms. Experiments were repeated three times.
[b] Biomass was evaluated as the dry weight of 10 tomato plants ± standard deviation compared with that of control plants (100%). The experiment was repeated three times.
[c] EPS produced in a 12-day culture. NT, not tested.

exoenzymes directed against plant cell wall components constitute important virulence factors (4, 6, 21, 23), strain NCPPB382 and its derivatives were tested for the production of endocellulase, polygalacturonase, and xylanase by standard agar plate assays. Assays for polygalacturonase and xylanase activity were positive for all strains, while endocellulase activity was detected only in those strains harboring plasmid pCM1, i.e., in the parental strain NCPPB382 and CMM101 (pCM1) (Table 2 and Fig. 4), indicating that the endocellulase is a plasmid-encoded enzyme.

Cloning of plasmid-derived DNA fragments involved in pathogenicity. The notion that plasmids pCM1 and pCM2 may encode genes involved in pathogenicity can be supported by cloning restriction fragments into the plasmid-free apathogenic strain CMM100 in order to reconstitute a pathogenic phenotype. Initial experiments performed by using vector pDM100 (16) were not successful since the instability of this vector (up to 5% segregation per generation) caused extensive plasmid loss under nonselective conditions in the plant. Therefore, two new vectors were constructed, solving the problem of segregation and resulting in vectors pDM302 and pDM306, which are stably maintained in *C. michiganensis* subsp. *michiganensis*. Vector pDM302 (14 kb) is a derivative of vector pDM10 (16) with the neomycin resistance gene of Tn*5* (3), the chloramphenicol resistance gene of *Corynebacterium xerosis* (14), and a 8.5-kb fragment of plasmid pCM1 carrying functions for replication and plasmid stability (Fig. 5). In vector pDM306 (13.6 kb), chloramphenicol resistance has been exchanged for the gentamicin resistance gene of Tn*1696* (32).

DNA restriction fragments of plasmid pCM1 were cloned into *E. coli* Ec294 with the *E. coli-Clavibacter* shuttle vector pDM302. In the case of *Bgl*II fragments of pCM1, cloning was first done with the *E. coli* vector pSVB30 (2), and fragments were then inserted as *Eco*RI-*Hin*dIII fragments into pDM302. Restriction fragments of plasmid pCM2 were cloned by employing vector pDM306. The resulting hybrid plasmids carrying DNA fragments of either pCM1 or pCM2 were isolated from *E. coli* and then used to transform the plasmid-free strain CMM100.

Numerous transformed clones of CMM100 were tested for their ability to induce specific disease symptoms, i.e., leaf wilting. The ability to induce wilting was restored to the same extent as observed for CMM101 (pCM1) and CMM102 (pCM2) in those clones of CMM100 which carried either the hybrid plasmid pDM302:B1 with a 3.2-kb *Bgl*II fragment of plasmid pCM1 or plasmid pJD2107 with a 3.75-kb *Bgl*II fragment of plasmid pCM2 (Table 2). Furthermore, we observed that pDM302:B1 carries the gene for an endocellulase (Table 2 and Fig. 4) which may represent the patho-

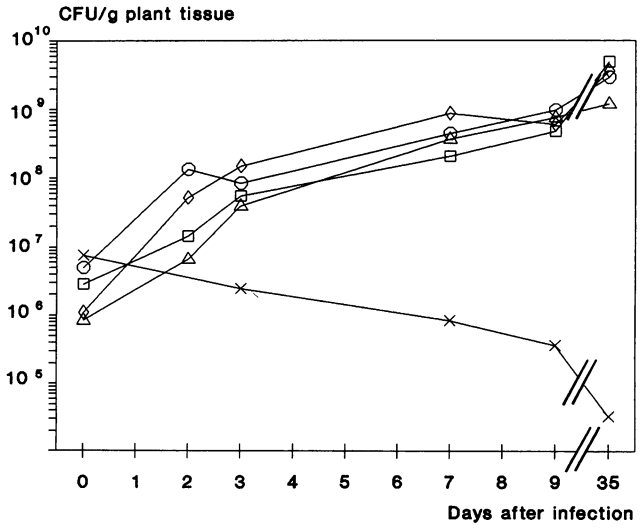

FIG. 3. Bacterial titers in planta at different times after infection. ○, NCPPB382; △, CMM100; ◇, CMM101; □, CMM102; ×, NCPPB3123.

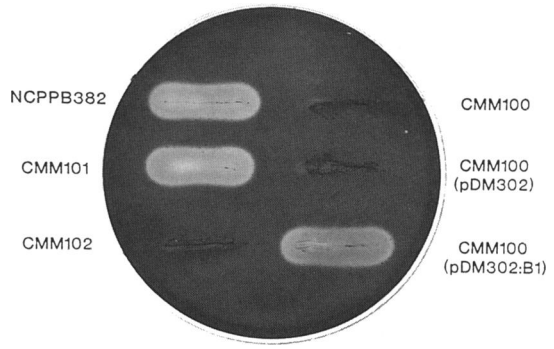

FIG. 4. Endocellulase production by different strains assayed on agar plates and stained with Congo red, as described in Materials and Methods. The halos around the bacterial streaks indicate endocellulase production.

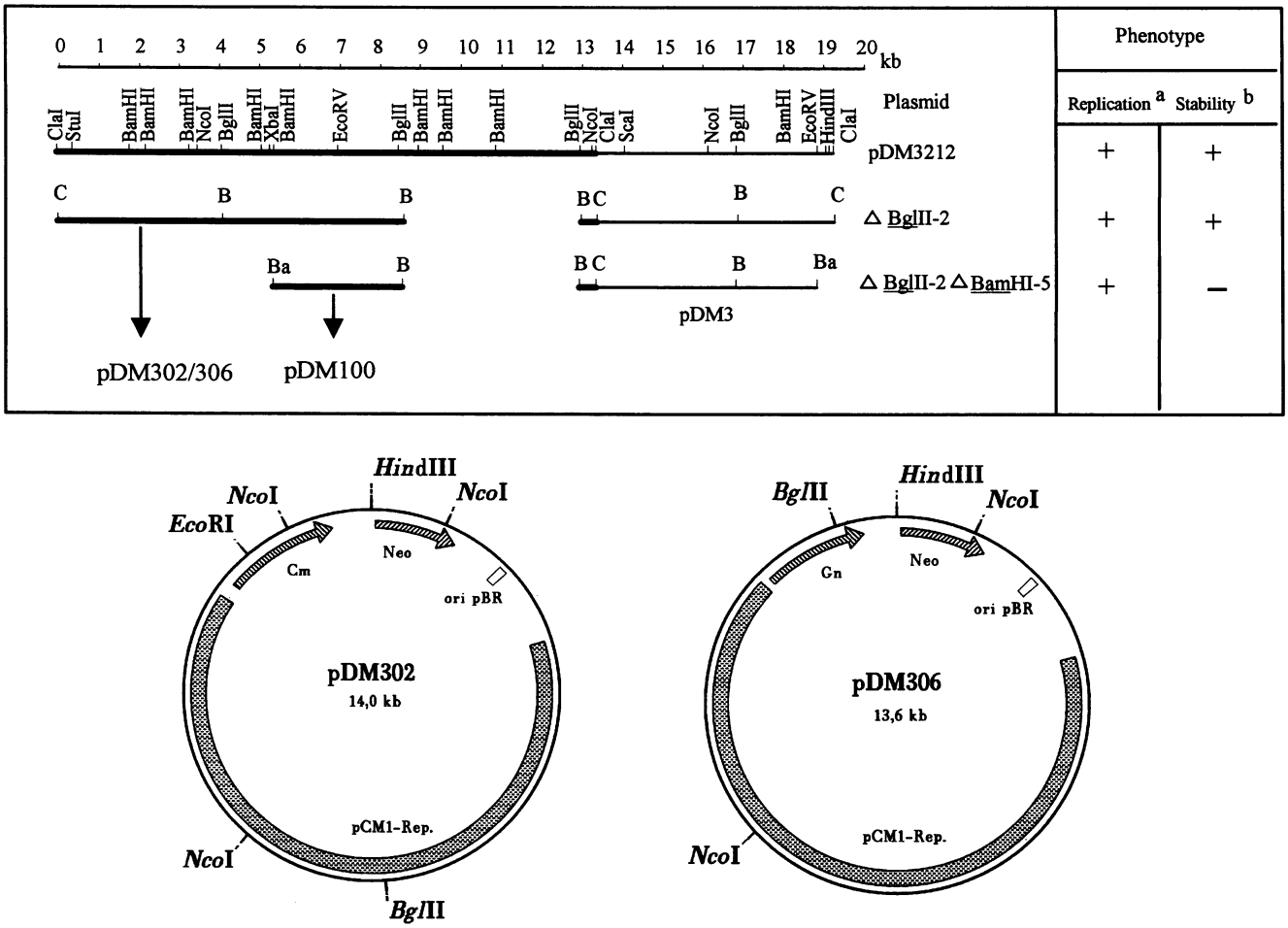

FIG. 5. Physical maps and properties of *E. coli-Clavibacter* shuttle vectors pDM302 and pDM306. The upper panel shows the restriction map of the region carrying the replicon of pCM1. Thick lines represent the regions of pCM1 DNA present in vectors pDM302 and pDM306 compared with those of the previously described vector pDM100 (16). For replication, + indicates that the plasmid was established in strain CMM100 by electroporation, as reported previously (16). For stability, + indicates that no segregation was observed during growth under nonselective conditions for 30 generations; − indicates about 5% segregation per generation. Neo, neomycin phosphotransferase; Gn, gentamicin acetyltransferase; Cm, chloramphenicol resistance gene of *C. xerosis* (14); ori pBR, origin of replication of vector pBR322; pCM1-Rep., replicon region of plasmid pCM1.

genic determinant expressed by this hybrid plasmid. In Fig. 1, the positions of those fragments on the plasmid maps are indicated. These data present clear evidence for the involvement of plasmids pCM1 and pCM2 in the pathogenic interaction of strain NCPPB382 with the host plant.

DISCUSSION

C. michiganensis subsp. *michiganensis* causes a vascular infection of tomato plants which results in wilting and wasting of the plant. A massive colonization of xylem vessels by the bacteria in conjunction with the production of high-molecular-weight EPS provides, at first glance, a simple explanation for this phenomenon, since it can easily be envisioned that these may drastically impair water transport. Furthermore, EPS have been proposed to be phytotoxins (19, 20) since tomato cuttings start to wilt when exposed to the EPS. But, this kind of test seems to be rather unspecific, as pointed out by Van Alfen and MacMillan (26), since many other compounds, even bovine serum albumin, give the same reaction; so, EPS are better classified as a phytoaggressin (12). Surprisingly, our data obtained with strain CMM100 show that colonization and EPS production were not sufficient for wilt induction but that the development of disease symptoms requires additional, plasmid-encoded gene products.

In the past, attempts to correlate plasmids to virulence in the genus *Clavibacter* have failed (5, 17, 18, 30); therefore, it was possibly a coincidence that this was the case in strain NCPPB382, which we decided to use in our work. There are pathogenic strains of *C. michiganensis* subsp. *michiganensis* in which we detected no plasmids. However, we found hybridization signals when probing restricted total DNA of such strains with labeled *Bgl*II fragments of plasmids pDM302:B1 and pJD2107 (15a), indicating that in plasmid-free pathogens the genetic information contained on these fragments is present but located on the chromosome.

A strain cured of both plasmids (CMM100) completely lost the ability to induce wilting of an infected plant, and when only one of the two plasmids is present, reduced virulence and a marked retardation in the onset of wilting are observed. This indicates that plasmid functions important for

pathogenicity act independently and that several different factors are involved.

By using vectors pDM302 and pDM306, two plasmid regions which carry pathogenic determinants of plasmids pCM1 and pCM2 were cloned. So far, we have no information on the nature of the pathogenic determinant of plasmid pCM2 carried by the hybrid plasmid pJD2107. In the case of pCM1, however, it seems likely that the endocellulase gene present on pDM302:B1 contributes to the development of the wilting disease, but it is also entirely possible that another gene locus present on the insert is involved. Work is being initiated to further characterize the cloned fragments in order to eventually understand the mechanism for the induction of bacterial wilt in the tomato by *C. michiganensis* subsp. *michiganensis*.

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REFERENCES

1. **Andro, T., J.-P. Chambost, A. Kotoujansky, J. Cattaneo, Y. Bertheau, F. Barras, F. Van Gijsegem, and A. Coleno.** 1984. Mutants of *Erwinia chrysanthemi* effective in secretion of pectinase and cellulase. J. Bacteriol. **160:**1199–1203.
2. **Arnold, W., and A. Pühler.** 1988. A family of high-copy-number plasmid vectors with single end-label sites for rapid nucleotide sequencing. Gene **70:**171–179.
3. **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 Tn*5*. Gene **19:** 327–336.
4. **Benhamou, N.** 1991. Cell surface interactions between tomato and *Clavibacter michiganense* subsp. *michiganense*: localization of some polysaccharides and hydroxyproline-rich glycoproteins in infected host leaf tissues. Physiol. Mol. Plant Pathol. **38:**15–38.

4a. **Bermpohl, A., and R. Eichenlaub.** Unpublished data.

5. **Clark, C. M., and C. H. Lawrence.** 1986. Characterization of a plasmid in isolates of *Corynebacterium sepedonicum*. Can. J. Microbiol. **32:**617–622.
6. **Collmer, A., and N. T. Keen.** 1986. The role of pectic enzymes in plant pathogenesis. Annu. Rev. Phytopathol. **24:**383–409.
7. **Collmer, A., J. L. Ried, and M. S. Mount.** 1988. Assay methods for pectic enzymes. Methods Enzymol. **161:**329–335.
8. **Davis, M. J., A. G. Gillaspie, Jr., A. K. Vidaver, and R. W. Harris.** 1984. *Clavibacter*: a new genus containing some phytopathogenic coryneform bacteria, including *Clavibacter xyli* subsp. *xyli* sp. nov., subsp. nov. and *Clavibacter xyli* subsp. *cyonodontis* subsp. nov., pathogens that cause ratoon stunting disease of sugarcane and bermudagrass stunting disease. Int. J. Syst. Bacteriol. **34:**107–117.
9. **Dische, Z.** 1962. Color reactions of carbohydrates. Methods Carbohydr. Chem. **1:**478–514.
10. **Fatmi, M., and N. W. Schaad.** 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology **78:**121–126.
11. **Ghangas, G. S., Y.-J. Hu, and D. B. Wilson.** 1989. Cloning of a *Thermomonospora fusca* xylanase gene and its expression in *Escherichia coli* and *Streptomyces lividans*. J. Bacteriol. **171:** 2963–2969.
12. **Graniti, A.** 1972. The evolution of the toxin concept in plant pathology, p. 1–18. *In* R. K. S. Wood, A. Ballio, and A. Graniti (ed.), Phytotoxins in plant diseases. Academic Press, London.
13. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
14. **Kassing, F.** Unpublished data.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

15a. **Meletzus, D., J. Dreier, and R. Eichenlaub.** Unpublished data.

16. **Meletzus, D., and R. Eichenlaub.** 1991. Transformation of the phytopathogenic bacterium *Clavibacter michiganense* subsp. *michiganense* by electroporation and development of a cloning vector. J. Bacteriol. **173:**184–190.
17. **Mogen, B. D., and A. E. Oleson.** 1987. Homology of pCS1 plasmid sequences with chromosomal DNA in *Clavibacter michiganense* subsp. *sepedonicum*: evidence for the presence of a repeated sequence and plasmid integration. Appl. Environ. Microbiol. **53:**2476–2481.
18. **Mogen, B. D., A. E. Oleson, R. B. Sparks, N. C. Gudmestad, and G. A. Secor.** 1988. Distribution and partial characterization of pCS1, a highly conserved plasmid present in *Clavibacter michiganense* subsp. *sepedonicum*. Phytopathology **78:**1381–1386.
19. **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.
20. **Rai, P. V., and G. A. Strobel.** 1968. Phytotoxic glycopeptides produced by *Corynebacterium michiganense*. II. Biological properties. Phytopathology **59:**53–57.
21. **Roberts, D. P., T. P. Denny, and M. A. Schell.** 1988. Cloning of the *egl* gene of *Pseudomonas solanacearum* and analysis of its role in phytopathogenicity. J. Bacteriol. **170:**1445–1451.
22. **Rothwell, A.** 1968. Bacterial canker of tomato caused by *Corynebacterium michiganense* (Smith) Jensen. Rhod. Agric. J. **1871:**75–78.
23. **Schell, M. A., D. P. Roberts, and T. P. Denny.** 1988. Analysis of the *Pseudomonas solanacearum* polygalacturonase encoded by *pgl*A and its involvement in phytopathogenicity. J. Bacteriol. **170:**4501–4508.
24. **Strobel, G. A.** 1970. A phytotoxic glycopeptide from potato plants infected with *Corynebacterium sepedonicum*. J. Biol. Chem. **245:**32–38.
25. **Van Alfen, N. K.** 1989. Reassessment of plant wilt toxins. Annu. Rev. Phytopathol. **27:**533–550.
26. **Van Alfen, N. K., and B. D. MacMillan.** 1982. Macromolecular plant-wilting toxins: artifacts of the bioassay method? Phytopathology **72:**132–135.
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. **Van Den Bulk, R. W., H. J. M. Löffler, and J. J. M. Dons.** 1990. Inhibition of callus development from protoplasts of *Lycopersicon peruvianum* by extracellular polysaccharides of *Clavibacter michiganensis* subsp. *michiganensis*. Plant Sci. **71:**105–112.
29. **Van Steekelenburg, N. A. M.** 1985. Resistance to *Corynebacterium michiganense* in tomato genotypes. Euphytica **34:**245–250.
30. **Vidaver, A. K.** 1982. The plant pathogenic corynebacteria. Annu. Rev. Microbiol. **36:**495–517.
31. **Wallis, F. M.** 1977. Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense*. Physiol. Plant Pathol. **11:**333–342.
32. **Wohlleben, W., W. Arnold, L. Bissonnette, A. Pelletier, A. Tanguay, P. H. Roy, G. C. Gamboa, G. F. Barry, E. Aubert, J. Davies, and S. A. Kagan.** 1989. On the evolution of Tn*21*-like multiresistance transposons: sequence analysis of the gene (*aac*C1) for gentamicin acetyltransferase-3-I (AAC(3)-I), another member of the Tn*21*-based expression cassette. Mol. Gen. Genet. **217:**202–208.