The Endo- β -1,4-glucanase CelA of *Clavibacter michiganensis* subsp. *michiganensis* Is a Pathogenicity Determinant Required for Induction of Bacterial Wilt of Tomato

Holger Jahr, Jens Dreier, Dietmar Meletzus, Rainer Bahro, and Rudolf Eichenlaub

Universität Bielefeld, Fakultät für Biologie, Gentechnologie/Mikrobiologie, D-33501 Bielefeld, Germany

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The phytopathogenic bacterium *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382, which causes bacterial wilt and canker of tomato, harbors two plasmids, pCM1 (27.35 kb) and pCM2 (72 kb), encoding genes involved in virulence (D. Meletzus, A. Bermpohl, J. Dreier, and R. Eichenlaub, 1993, J. Bacteriol. 175:2131-2136; J. Dreier, D. Meletzus, and R. Eichenlaub, 1997, Mol. Plant-Microbe Interact. 10:195-206). The region of pCM1 carrying the endoglucanase gene *celA* was mapped by deletion analysis and complementation. RNA hybridization identified a 2.4-knt (kilonucleotide) transcript of the *celA* structural gene and the transcriptional initiation site was mapped. The *celA* gene encodes CelA, a protein of 78 kDa (746 amino acids) with similarity to endo- β -1,4-glucanases of family A₁ cellulases. CelA has a three-domain structure with a catalytic domain, a type IIa-like cellulose-binding domain, and a C-terminal domain. We present evidence that CelA plays a major role in pathogenicity, since wilt induction capability is obtained by endoglucanase expression in plasmid-free, nonvirulent strains and by complementation of the CelA⁻ gene-replacement mutant CMM-H4 with the wild-type *celA* gene.

Additional keywords: gram-positive bacterium.

Clavibacter michiganensis subsp. *michiganensis* (Cmm), which causes bacterial wilt and canker of tomato (*Lycopersicon esculentum* Mill.) (Davis et al. 1984), is a gram-positive bacterium which that subject to quarantine regulations in the European Community as well as internationally (European Union 1995). The disease causes severe crop failures in all main cultivation areas (Shirakawa et al. 1991). A latent infection, resulting from inoculation at a late stage of plant development,

may also be destructive by generating contaminated transplants or seed (Gitaitis et al. 1991; Fatmi and Schaad 1988; Thyr 1968). At present, neither resistant tomato cultivars nor effective chemical controls of this pathogen are known (Thompson 1986), but sensitive and highly specific screening techniques have been reported recently that can be used for certification of seed or transplants (Santos et al. 1997; Dreier et al. 1995).

Three mechanisms have been proposed to explain the wilting disease caused by the vascular phytopathogen *C. michiganensis*: mechanical plugging of xylem vessels; toxic action of exopolysaccharides (EPS); and enzymatic attack on plant tissue (reviewed by Jahr et al. 1999). In several studies, plugging of xylem vessels by high titer colonization and production of high molecular weight EPS could be ruled out as the primary reason for the onset of plant wilting (Bermpohl et al. 1996; Meletzus et al. 1993). Spreading of bacteria in tissue of young tomato plants and progressive tissue breakdown during initial stages of disease development have been shown (Wallis 1977). In early stages of infection, Cmm colonizes the xylem vessels, while colonization of the phloem, pith, and cortex of the upper parts of the plant is only observed at later stages. Wilting may therefore result from an enzymatic attack on xylem vessels and adjacent parenchymatic cells (Benhamou 1991; Wallis 1977), allowing a lateral spread of Cmm within the host plant, along with the release of nutrients necessary for bacterial proliferation and colonization.

Bacterial vascular diseases are caused by several genera of bacteria, such as *Erwinia*, *Ralstonia*, *Xanthomonas*, and *Clavibacter*. In *Erwinia carotovora* subsp. *carotovora* (Walker et al. 1994; Mäe et al. 1995), endoglucanase-defective mutants were less virulent. In *Ralstonia solanacearum*, however, in addition to EPS and polygalacturonase, an extracellular endoglucanase is necessary for full virulence (Allen et al. 1997) but not essential for symptom development, as shown by site-directed mutagenesis (Roberts et al. 1988). In the genus *Clavibacter*, production of extracellular enzymes such as endoglucanase (Meletzus et al. 1993), pectinmethylesterase (Strider 1969), polygalacturonase, and xylanase (Beimen et al. 1992) has been reported and endoglucanase activity has been implicated as a pathogenicity factor in recent reports (Jahr et al. 1999; Metzler et al. 1997).

For Cmm NCPPB382, it has been shown that genes encoded by the endogenous plasmids pCM1 and pCM2 are cru-

Corresponding author: Rudolf Eichenlaub; Universität Bielefeld, Gentechnologie/Mikrobiologie, D-33501 Bielefeld, Germany
Telephone: (+49) 521-106-5558; Fax: (+49) 521-106-6015
E-mail: eichenlaub@biologie.uni-bielefeld.de

Current address of Jens Dreier: Herz- und Diabeteszentrum NRW, Inst. für Laboratoriums- und Transfusionsmedizin, Georgstr. 11, D-32545 Bad Oeynhausen, Germany.

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cial for virulence (Dreier et al. 1997; Meletzus et al. 1993). Recently, we described the pathogenicity locus *pat-1* carried by pCM2 (Dreier et al. 1997). In the present study, we demonstrate that the *celA* gene encoding an endoglucanase that maps on pCM1 (Meletzus et al. 1993) is required for the development of disease symptoms on infected tomato plants.

RESULTS

Nucleotide sequence of the *celA* gene and deduced amino acid sequence of CelA.

It was shown previously that in *Cmm* NCPPB382 the loss of plasmids pCM1 and pCM2 results in the avirulent endophytic, endoglucanase-negative derivative CMM100, lacking wilt-inducing ability (Meletzus et al. 1993). Reduced virulence was observed for the partially cured derivative CMM101, carrying only pCM1 (Fig. 1), which produced endoglucanase and caused wilting of tomato plants with a delay of 6 days, compared with the parental strain NCPPB382 (Meletzus et al. 1993). It was also shown (Meletzus et al.

1993) that the 3.2-kb *Bgl*II restriction fragment B1 of plasmid pCM1 carries an endoglucanase gene. Transformation of the plasmid-free strain CMM100 with hybrid plasmid pDM302:B1 restored endoglucanase activity in plate assays with carboxymethylcellulose (CMC) and wilt induction in infected tomato plants.

To determine the nucleotide sequence of the *celA* gene, the *Bgl*II-1 restriction fragment of pCM1 was inserted into the single *Bam*HI restriction site of *Escherichia coli* sequencing vector pSVB30 (Arnold and Pühler 1988). Analysis of the sequence revealed the presence of one open reading frame from GTG₅₃₅ to the termination codon TGA₂₇₇₃ preceded by two putative ribosome binding sites (RBS) at nucleotide positions 527 and 539, respectively (Fig. 2). The first RBS showed significant homology to such sites of genes known to be expressed in the genus *Clavibacter* as well as to RBS from streptomycetes (collected by Strohl 1992) and some closely related, gram-positive bacteria (data not shown). The second putative RBS reveals significant complementarity to the 3' end of *Clavibacter* spp. 16S rRNA (data not shown). If

Fig. 1. Map of plasmid pCM1. Restriction enzyme recognition sites not unique to pCM1, indicated by asterisks. *Bgl*II-1 fragment and endo- β -1,4-glucanase gene *celA*, indicated by black box and arrow, respectively. Regions essential for plasmid replication and stability, indicated as a bold-lined or thin-lined open box and labeled.

GTG₅₃₅ is used as the start codon, the deduced protein contains 746 amino acid (aa) residues and has a molecular mass of 78,000 Da.

The first 51 aa residues of the N terminus of the protein resemble a signal sequence with a positively charged N-terminal region composed of basic amino acids (positions 2 and 3, arginine and lysine), followed by a nonpolar, hydrophobic central part mainly composed of alanine, leucine, and valine, and a short C-terminal polar region (serine and threonine). Positively charged amino acids further occur at amino acid positions 25, 28, 34. The putative cleavage site for a signal peptidase, determined according to the -3, -1 rule of von Heijne (1986), is located between the two alanines at amino acid sequence positions 51 and 52. Processing would result in a mature protein with a molecular mass of approximately 72 kDa. Further sequence analysis revealed a modular structure of the mature protein, typical for cellulases with a catalytic domain of approximately 350 aa and a nearly perfect type IIa bacterial cellulose-binding domain (CBD) of 84 aa, connected by a 27-aa linker region primarily consisting of serine and proline residues, typical for hinge regions (Gilkes et al. 1991).

All known family A enzymes are, with the exception of the β -1,4-mannanase from *Caldocellum saccharolyticum* (Henrissat 1991), endo- β -1,4-glucanases and have a catalytic domain of 350 to 500 aa (Meinke et al. 1993). The catalytic domain of CelA is most significantly similar to β -1,4-endoglucanases of *Acidothermus cellulolyticus* (E1; accession no. U33212), *Bacillus polymyxa* (Baird et al. 1990), *Xanthomonas campestris* pv. *campestris* (EngXCA; Gough et al. 1990), and *C. saccharolyticum* (CelB; Saul et al. 1989), with amino acid

identities of 51.9, 41.9, and 33% over the entire domain (Fig. 3). Based on these similarities, the catalytic domain of CelA can be grouped into subtype 1 of glycosyl hydrolase family 5 (synonym: cellulase family A₁; Henrissat 1991). Furthermore, CelA shows four conserved motifs (GNP, VIH, NPG, and NHY) with seven invariant amino acid residues in the active center (Ducros et al. 1995; Henrissat and Bairoch 1993; Henrissat et al. 1989), R128, H178, N223, E224, H301, Y303, and E345 with respect to the presumed translational start site GTG₅₃₅. Glutamic acid E345 corresponds to the active site nucleophile (Wang et al. 1993) of this "retaining" glycosidase (Withers et al. 1990), possibly interacting with putative proton donor E224 (Fig. 3A).

The catalytic domain is connected to a type IIa CBD (Tomme et al. 1995) via a short hinge region (Gilkes et al. 1991). Multiple sequence alignment (Fig. 3B) revealed overall identities of up to 35% with the most closely related members of this CBD family. The similarity is imperfect, since CelA is lacking two cysteines typically flanking the CBD that are supposed to be involved in formation of a disulfide bridge (Gilkes et al. 1991). Several other CBDs of this family, however, also lack these cysteines (Tomme et al. 1995). Three highly conserved tryptophan residues that are involved in substrate binding (Tormo et al. 1996) are also found in CelA within the conserved motifs designated WGN and WGG (indicated in Figure 3B by asterisks).

Surprisingly, and as a unique feature, the CBD of CelA is followed by a 200 aa C terminus (Fig. 4) that is connected to the CBD through a second 28 aa linker, indicating that the C terminus of CelA may harbor an additional domain, the func-

Fig. 2. Partial nucleotide sequence of the *celA* locus. Direct (DR) and inverted (IR) repeats, shown by arrows. Putative promoter, indicated by "-35"/"-10"; startpoint of transcription, indicated by arrow and "+1". Putative Shine-Dalgarno sites (SD) are shaded in black; corresponding putative translational start sites (GTG) are underlined. Possible cleavage site of signal peptide, indicated by a vertical arrowhead.

A

B

Fig. 3. Amino acid sequence comparison of part of catalytic domains of (A) family 5 glycosyl hydrolases and (B) family II cellulose-binding domains (CBDs). Sequences with the most significant similarity to *Cela*: *BpoCel*, *Bacillus polymyxa* endo- β -1,4-glucanase (Baird et al. 1990); *BfiEnd1*, *Butyrivibrio fibrisolvens* endoglucanase 1 (Berger et al. 1989); *CsaCelB*, *Caldocellum saccharolyticum* endo- β -1,4-glucanase B (Saul et al. 1989); *CfiCenD*, *Cellulomonas fimi* β -1,4-endoglucanase B, D (Meinke et al. 1991, 1993); *PflEgA*, *Pseudomonas fluorescens* subsp. *cellulosa* endoglucanase A (Hall and Gilbert 1988); *PflXynB/C*, *P. fluorescens* subsp. *cellulosa* xylanases B, C (Kellett et al. 1990, identical sequences); *SliCela*, *Streptomyces lividans* (Thberge et al. 1992); *XcaEng*, *Xanthomonas campestris* endo- β -1,4-glucanase (Gough et al. 1990). Identical amino acid residues are boxed in black; similar residues are boxed in gray. Consensus line points out highly conserved residues by asterisks within black-boxed BLOCKS motifs GNP, VIH, NPG, and NHY (BL00659A-D). B, Highly conserved tryptophan residues of (putative) CBDs indicated by "#"; arrows indicate conserved CBD-flanking cysteine residues. BLOCKS motifs WGN and WGG (BL00561A-B) are indicated.

tion of which so far remains unclear. A data base search provided a weak homology to α -expansins, plant proteins mediating cell wall extension (Cosgrove 1998).

Transcription analysis of *celA*.

To investigate expression of the *celA* gene, the length of the *celA* mRNA was determined by Northern (RNA) hybridization with an RNA probe complementary to the *celA* mRNA. Total RNA was isolated from cells of CMM100 and CMM101(pCM1) and subjected to Northern hybridization (Fig. 5). A single RNA transcript of approximately 2,400 nucleotides was detected in strain CMM101(pCM1). No such transcript was observed in the plasmid-free CMM100 control. The 5' end of *celA* mRNA was mapped by primer extension. The start of transcription, a cytosine residue, was located 85 nucleotides upstream of the translational start codon GTG₅₃₅.

SDS-PAGE and activity staining of CelA.

To demonstrate the functional expression of CelA, concentrated culture supernatants of strains CMM101(pCM1) and CMM100(pHJ1200) (Fig. 6) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The CMC-containing gels were stained with Congo red. The supernatant of CMM101(pCM1) gave rise to three bands with CMCase activity and molecular masses of approximately 75,

Fig. 5. Analysis of *celA* RNA by Northern (RNA) hybridization. On left, stained agarose gel with RNA extracted from strains CMM101(pCM1) and CMM100 with 23S and 16S rRNAs as prominent bands. On right, digoxigenin (DIG)-labeled RNA marker (lane 1, size in knt is indicated). Hybridization with the *celA* probe (see Materials and Methods) identifies a 2.4-knt, *celA*-specific transcript indicated by arrow (lane 2). This transcript is missing in the plasmid-free, *celA*-negative strain CMM100 (lane 3).

Fig. 4. Phenotypes of *celA* deletion clones. All derivatives of the *Bgl*III-1 fragment of pCM1 (shown on top) were inserted into vector pDM302. Only relevant restriction enzyme recognition sites are indicated with corresponding nt-positions. *celA* gene indicated by arrow; gene product CelA with the three-domain structure is shown below (SP = signal peptide), as is the integration site and orientation of antibiotic resistance cassette *aacC1* in *celA*. Plasmid names, corresponding endoglucanase activity (CMCase activity), and pathogenic phenotype when transformed into CMM100, shown on right.

54, and 43 kDa. The native protein after processing would have a theoretical molecular mass of 72 kDa, corresponding to the band of slowest mobility. The molecular masses of the other two active bands are in agreement with a cleavage of the intact CelA protein within the hinge regions. Such cleavage would result in a protein of about 52 kDa, consisting of the catalytic domain and the CBD but lacking the C-terminal domain, and a protein of approximately 42 kDa containing only the catalytic domain. CMCase activity of such truncated enzymes is supported by the enzymatic activity of CMM100(pHJ1200) (Fig. 6, lane 3) and other deletion mutants (Fig. 4).

The *celA* gene product is a wilt-inducing determinant.

As mentioned above, introduction of hybrid plasmid pDM302:B1 into CMM100, the nonvirulent, plasmid-free derivative of NCPPB382, restored the virulent, wilt-inducing phenotype (Meletzus et al. 1993). Conversion to the wilt-inducing phenotype was also observed when the natural isolate CMM71-S, which has no *celA* gene (Dreier et al. 1997), was transformed with hybrid plasmid pDM302:B1 (Table 1), indicating that the *celA* gene is a pathogenicity determinant.

To confirm this notion, hybrid plasmids with various deletions of the 3.2-kb fragment were constructed and introduced into strain CMM100. Figure 4 presents the results of CMCase activity tests and virulence assays. Loss of the C-terminal CBD abolishes wilt induction, but not the CMCase activity of the truncated CelA encoded by pHJ102. Neither the N-terminal region nor the C-terminal part of CelA are sufficient for CMCase activity or wilt induction in tomato plants (pHJ104, pHJ105). A partial deletion of the putative "third domain" does not affect CMCase activity but wilt induction is lost. Thus, only intact CelA (pHJ1010) with the CBD and the C-terminal "third domain" is able to induce disease symptoms in infected tomato plants.

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of CelA and zymogram. Concentrated culture supernatant of strain CMM101(pCM1) (lane 2) and strain CMM100(pHJ1200) (lane 3) and a prestained molecular weight marker (lane 1) were subjected to SDS-PAGE and stained with Congo red. The three active CMCase species with 75, 54, and 43 kDa are indicated. Strain CMM100(pHJ1200) expresses only the 43-kDa enzyme representing the catalytic domain of CelA.

Construction of a *celA* gene replacement mutant.

To address the question of whether the native plasmid pCM1 carries genes in addition to *celA* that are involved in wilt induction in the host plant, an interposon mutation of *celA* was constructed for a gene replacement experiment. The 3.2-kb *Bgl*II-1 fragment was inserted into the unique *Bam*HI restriction site of vector pSVB30, generating hybrid plasmid pHJ-1. The *celA* gene was then disrupted by insertion of a 1.6-kb *Sma*I fragment of plasmid pHN5 (Laine et al. 1996), carrying the gentamicin acetyltransferase gene *aacC1* (Wohlleben et al. 1989), into the unique *Bsp*120I site of pHJ10, after fill-in of the 3' recessed ends (Fig. 4). The resulting hybrid plasmid pHJ-III (Fig. 4) carried the antibiotic resistance cassette at nucleotide position 716, disrupting the *celA* structural gene. This plasmid was then used for subsequent transformation of CMM101(pCM1), where it cannot replicate, and antibiotic resistant clones arising on Luria-Bertani plates with gentamicin were screened for lack of CMCase activity. To confirm the correct integration of the *aacC1* cassette in pCM1, Southern hybridization was performed and the mutant CMM-H4 was found to carry the cassette as a result of a double crossover event.

A *celA*-specific, 3.2-kb signal (Dreier et al. 1995) was detected in Southern hybridization with *Bgl*III-digested plasmid and total DNA of CMM101 with a *celA* probe (Fig. 7A, lanes 5 and 8), but not in the mutant CMM-H4, where two signals corresponding to DNA fragments of approximately 1.3 and 3.55 kb (Fig. 7A, lanes 4 and 7; Fig. 4) appeared. Furthermore, an *aacC1*-specific probe was used, resulting in the same hybridization patterns obtained with the *celA*-specific probe (data not shown). Thus, the two *Bgl*III-restriction fragments of 1,260 and 3,544 bp hybridizing with the *celA*-specific probe confirm the presence of the *aacC1* cassette in the *celA* gene of mutant CMM-H4 as the result of a double crossover event.

In virulence assays the gene replacement mutant CMM-H4 had lost the ability to induce wilting in infected tomato plants (Table 1), demonstrating that *celA* is the only pathogenicity gene carried by plasmid pCM1. Complementation of the *celA::aacC1* gene by introduction of pDM302:B1 into CMM-H4 restored the CMCase activity and the ability to induce

Table 1. Phytopathogenic phenotype conferred by various plasmid constructs

Strain	Plasmid status ^a	Wilting index ^b	Biomass ^c
CMM101	pCM1	10.5 (± 0.5)	58 (± 24)
CMM100	—	—	78 (± 16)
CMM100	pDM302	—	68 (± 17)
CMM100	pDM302:B1	16.0 (± 1.0)	63 (± 19)
CMM100	pHJ1010	17.0	63 (± 19)
CMM100	pHJ1103	—	65 (± 15)
CMM100	pHJ1102	—	65 (± 18)
CMM-H4	pCM1- <i>celA::aacC1</i>	—	NT ^d
CMM-H4c	pCM1- <i>celA::aacC1</i> (pDM302:B1)	12 (± 1.0)	NT
CMM71-S	—	—	93 (± 20)
CMM71-S	pDM302	—	90 (± 18)
CMM71-S	pDM302:B1	27	NT

^a For maps of plasmids see Figures 1 and 4.

^b Values in parentheses represent wilting index, number of days when 50% of infected plants (*n* = 30) showed wilting symptoms.

^c Expressed as percentage of dry weight of 10 tomato plants, 4 weeks after inoculation, compared with uninfected control plants (100%).

^d Not tested.

wilting (Table 1). This strain, CMM-H4c, was also analyzed by Southern hybridization (Fig. 7B, lane 5) and as expected an additional signal of 15.6 kb was obtained that represents the linearized vector pDM302:B1 corresponding to the pDM302:B1 control (Fig. 7B, lane 6). Thus, in strain CMM-H4c the complementing plasmid pDM302:B1 was present. Loss of virulence as a consequence of inactivation of the *celA* gene by interposon mutagenesis and consecutive restoration of virulence by complementation with the intact *celA* gene demonstrates that *celA* is the only pathogenicity determinant carried by pCM1.

DISCUSSION

In this study we analyzed the *celA* gene of *Cmm* encoding the endo- β -1,4-glucanase CelA required for the pathogenic phenotype mediated by endogenous plasmid pCM1 of strain NCPPB382. The nucleotide sequence of the *Bgl*II-1 fragment revealed one open reading frame of 2,238 bp and a corresponding mRNA of approximately 2.4 knt (kilonucleotides) for *celA* was identified by Northern hybridization. The transcriptional initiation site was mapped by primer extension. Comparison of the nucleotide sequence upstream of the *celA* gene with known promoters from other G+C-rich, gram-positive bacteria (e.g., *Corynebacterium glutamicum*; Pátek et al. 1996) and *Clavibacter xyli* subsp. *cynodontis* (Haapalainen et al. 1996) did not reveal sufficient homology to determine a putative promoter element. Several direct and one inverted

repeat could be identified in the 5' region of the *celA* gene (Fig. 2), and a possible regulatory role in gene expression of such structural elements has been discussed previously (Dreier et al. 1997; Delic et al. 1992; Vidal-Ingigliardi et al. 1991).

Putative RBS preceding the *celA* gene were identified at -4 bp (5'-AGGAG-3') and -7 bp (5'-GAAAGG-3') with respect to the putative translational start sites GTG₅₃₅ and GTG₅₅₀, respectively. Both revealed complementarity to the conserved 3' end of 16S rRNAs from the genus *Clavibacter* (Li and De Boer 1995; Sathyamoorthy et al. 1991) and are nearly identical to the consensus Shine-Dalgarno sequence of streptomycetes as reported by Strohl (1992). Furthermore, similar RBS were predicted for several cellulase genes of the closely related actinomycete *Cellulomonas fimi* (Meinke et al. 1993, 1991; Greenberg et al. 1987).

Extracellular enzymes are generally secreted by a two-step, Sec- and ATP-dependent, type II mechanism involving a signal peptidase (Salmond 1994). A putative signal peptide of 51 aa can be deduced for CelA from hydropathy plots (Kyte and Doolittle 1982), indicating that this enzyme is secreted via the type II mechanism.

The role of CelA in the phytopathogenic interaction of *Cmm* with its host plant *Lycopersicon esculentum* was demonstrated by transformation of the nonvirulent *Cmm* strains CMM100 and CMM71-S with vectors carrying the intact or truncated *celA* genes. In addition, the loss of pathogenicity resulting from inactivation of the *celA* gene locus on plasmid pCM1 in the nonvirulent mutant CMM-H4 by interposon mutagenesis, with a *celA::aacC1* construct (pHJ-III), was subsequently restored by complementation with the cloned intact *celA* gene. The delayed onset of wilting in CMM100(pHJ306) and CMM100(pDM302:B1), compared with strain CMM101(pCM1), may be an indication that pCM1 carries additional genes that affect colonization or utilization of nutrients but are not directly involved in the induction of wilting symptoms on the host plant.

Hydrolysis of cellulose requires a consortium of microbial enzymes (Béguin and Aubert 1994; Gilbert and Hazlewood 1993) and enzymatic attack on native cellulose is initiated at internal glucosidic bonds within an intact glucan chain by random attack of endo-type cellulases (EC 3.2.1.4), commonly creating nonreducing chain termini susceptible to attack of exo-type cellulases (EC 3.2.1.91) (Hoshino et al. 1997). CelA activity may, however, be sufficient for pathogenicity, although *Cmm* is not known to be a true cellulolytic bacterium. Plasmid pHJ1102, encoding a truncated enzyme that retained activity against amorphous CMC, did not confer a pathogenic phenotype when introduced into strain CMM100. Lack of the C-terminal region of the CBD in pHJ1102 suggests that a CBD is not required for CMCase activity of CelA. Expression of the catalytic domain from the hybrid plasmid pHJ1200 (Fig. 4) in strain CMM100 showed that this CelA fragment of about 42 kDa had CMCase activity (Fig. 6). Figure 6 (lane 2) shows that culture supernatant of CMM101(pCM1) contains three active forms of CelA with molecular masses of approximately 75, 54, and 43 kDa, indicating that CelA may be processed within the hinge regions. Such processing of extracellular enzymes is not unusual and has been reported for, e.g., pectate lyase PelI of *Erwinia chrysanthemi* (Shevchik et al. 1998), a *Streptomyces* sp. cellulase (Moormann et al. 1993) and chitinases (Vionis et al. 1996;

Fig. 7. Southern hybridization for analysis of gene replacement mutant CMM-H4. Restriction enzyme digested DNA was probed with a digoxigenin (DIG)-11-dUTP-labeled *celA* fragment. **A**, Nondigested plasmid pCM1 DNAs from CMM-H4 (lane 1) and CMM101 (lane 2), DIG-labeled *Eco*RI/*Hind*III-digested λ marker (lanes 3, 6), *Bgl*II-digested plasmid pCM1 DNAs from CMM-H4 (lane 4) and CMM101 (lane 5), and likewise digested total DNA from CMM-H4 (lane 7), CMM101 (lane 8), and gene replacement vector pHJ-III (lane 9). **B**, *Eco*RI/*Hind*III-digested λ marker (lanes 1, 4), *Bgl*II-digested total DNAs from CMM101 (lane 2), *celA*-mutant CMM-H4 (lane 3) and complemented mutant CMM-H4c (lane 5) and *Bgl*II-digested plasmid pDM302:B1 DNA (lane 6).

Blaak and Schrempf 1995), and endo- and exoglucanases of the actinomycete *Cellulomonas fimi* where cleavage at inter-domain junctions by specific extracellular proteases has been shown (Gilkes et al. 1989, 1988).

Type II CBDs from cellulases seem to increase the activity of catalytic domains against insoluble cellulose (Hall et al. 1995; Tomme et al. 1995; Coutinho et al. 1993), but the actual role of CBDs in cellulose hydrolysis is still insufficiently understood. They may simply increase effective enzyme concentration by altering substrate proximity (Bolan et al. 1998), or could facilitate the nonhydrolytic breakdown of crystalline cellulose more actively by disruption of its structure (Linder and Teeri 1997; Din et al. 1994, 1991). The CBD of CelA is a classical type IIa member according to amino acid sequence similarities, revealing conserved tryptophans (Poole et al. 1993) but lacking the two flanking cysteine residues (Fig. 3B). Analysis of deletions affecting the CBD and the third domain with respect to virulence indicates that the catalytic part of the enzyme is not sufficient for a pathogenic phenotype. Removal of the CBD may reduce the affinity for the native crystalline substrate, resulting in an accelerated degradation of small soluble substrates, as shown for *Streptomyces reticuli* Avicelase (Moormann et al. 1993) and fungal endoglucanases (Eriksson and Pettersson 1982).

Endoglucanases are known to be modular proteins with structurally distinct N- or C-terminal CBDs joined to catalytic domains (Linder and Teeri 1997; Gilkes et al. 1991). Sometimes there is found an additional domain like the fibronectin III-like domain of CenD (Meinke et al. 1993). CelA also has a three-domain structure. The only hint for a possible function of the "third domain" is a weak similarity to expansins of plant origin. Since hybrid plasmid pHJ1103 failed to restore the pathogenic phenotype in CMM100, this can be taken as evidence that the 200-aa, expansin-like "third domain" of CelA is required for virulence. The similarities to expansins were first identified for the endocellulase of *Clavibacter michiganensis* subsp. *sepedonicus* (M. Metzler, *personal communication*), which also exhibits a three-domain structure. Expansins are believed to act on different plant cell wall polymers, but share only few highly conserved residues, those necessary for protein-polysaccharide binding (Shcherban et al. 1995) and an H-F-D motif, also conserved in the active site of family K cellulases (family-45 glycosyl hydrolases) (Cosgrove 1998; Davies and Henrissat 1995). Similarities between the "third domain" and plant expansins are limited to a relatively cysteine-rich N-terminal region, a basic central region, and some conserved tryptophan residues near the C terminus. Since expansins are believed to break hydrogen bonds of carbohydrate polymers in native plant cell walls during growth, occurrence of an expansin-like domain within CelA may contribute to the hydrolytic activity of this endoglucanase expressed by the biotrophic pathogen *Cmm*. In addition to CelA, other extracellular enzymes such as polygalacturonase (Beimen et al. 1992), pectinmethylesterase (Strider 1969), and xylanase (Beimen et al. 1992) produced by *Cmm* may also be involved in the degradation of the composite polymeric cell wall structure. Thus, we propose that wilting is caused by hydrolytic degradation of xylem vessel walls by CelA resulting in an impairment of water transport by embolisms and lateral spread of bacteria into adjacent stem parenchyma accompanied by tissue maceration.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. For growth conditions and reisolation of bacteria see Meletzus and Eichenlaub (1991) and Dreier et al. (1997). Culture medium for RNA preparation was a modified Pettersson medium (pH 7.2) as described by Baer and Gudmestad (1995), designated CMBM, with addition of 0.1% (wt/vol) D-(+)-arabinose, 0.1% (wt/vol) yeast extract, 0.05% (wt/vol) Casamino Acids (no. 0288-01-2; Difco, Augsburg, Germany), 100 µg of thiamine hydrochloride per liter, 2 g of L-glutamine per liter.

RNA preparation.

Total RNA of *Cmm* strains was isolated as described by Sambrook et al. (1989) with modifications as reported by Dreier et al. (1997), except that the cells were grown up to a titer of approximately 5×10^8 cells per ml in CMBM medium, containing 0.1% (wt/vol) D-(+)-cellobiose (C-7252; Sigma, Deisenhofen, Germany), 0.2% CMC (Sigma, low viscosity) and 2% (vol/vol) tomato tissue extract. An average yield of about 2 mg of RNA was obtained from 50-ml cultures of *Cmm*.

CMCase plate assays, SDS-PAGE, and zymogram analysis.

Endoglucanase (CMCase) activity was detected on M9CMC agar plates (Meletzus et al. 1993), stained with 0.1% (wt/vol) Congo red for 15 to 30 min and finally bleached with QR-buffer (2 M NaCl, 50 mM Trizma base, pH 8.5). For SDS-PAGE the supernatant of 6-day-old cultures was rapidly harvested by centrifugation at $10,000 \times g$ for 20 min at 4°C and filtered at the same temperature through a polysulfone membrane with a molecular mass cut-off of 100 kDa, with an Amicon pressure cell 8400. After 50-fold concentration of proteins by 60% $(\text{NH}_4)_2\text{SO}_4$ precipitation, the supernatant was subjected to SDS-PAGE according to (Laemmli 1970), with modifications of Malburg and Forsberg (1993). Polyacrylamide gels (10%) contained 0.2% CMC and sample pretreatment was as described by Moormann et al. (1993), except that sample incubation was at 70°C. After electrophoresis, the gels were washed sequentially at room temperature once in 1%, twice in 0.1% Triton X-100, and twice in 20 mM Tris-HCl, pH 6.8, for 30 min each. Gels were stained with Congo red (Schwarz et al. 1987) after appropriate incubation at 37°C in sealed plastic bags. Prestained molecular mass marker was SeeBlue (NOVEX, San Diego, CA).

DNA sequencing.

DNA sequence was determined from supercoiled, double-stranded plasmid DNA for both strands by the dideoxy chain termination method (Sanger et al. 1977) with a Sequenase 2.0 sequencing kit according to the manufacturer's protocol (USB, Cleveland, OH), with $[\alpha^{32}\text{P}]\text{dATP}$ and $[\alpha^{35}\text{S}]\text{dATP}$ or nonradioactively on an A.L.F. sequencer (Pharmacia Biotech, Uppsala, Sweden). Sequence analyses were performed with computer software packages Microgenie (Beckman Instruments, Fullerton, CA) and PCgene (Intelligenetics, Geneva, Switzerland), respectively. Protein sequences deduced from the nucleotide sequence were compared with SwissProt sequence

data bases, with the BLAST tool, PROSITE and BLOCKS Database 9.3. Multiple sequence alignments were carried out with CLUSTAL W and shaded with BOXSHADE 3.21.

Northern transfer and hybridization.

Generally, RNA procedures were done according to the DIG system user's guide for filter hybridization (Boehringer Mannheim, Mannheim, Germany). An RNA probe, complementary to an internal part of *celA* mRNA, was derived from polymerase chain reaction (PCR)-amplified, 2.19-kb *celA* fragment, labeled according to the supplier's kit manual (DIG RNA Labeling Kit (SP6/T7); Boehringer Mannheim), with an *EcoRI*-digested pSPT19:*celA* vector to generate "run-off" anti-*celA* transcripts. Aliquots of 10 µg of total RNA were vacuum dried, resuspended in 10 µl of RNA buffer, denatured by warming to 68°C for 15 min, and snap cooled in ice water. Denatured RNAs were separated by formaldehyde agarose gel electrophoresis (Fourney et al. 1988), together with digoxigenin (DIG)-labeled RNA molecular weight marker I (Boehringer Mannheim). RNAs were transferred to Nytran-Plus membranes (Schleicher & Schuell, Dassel, Germany) by 16-h, upward capillary transfer (Southern 1979) and fixed with UV light. Prehybridizations and hybridizations were performed as described by Dreier et al. (1997) but at 68°C. Results were visualized with chemiluminescent substrate CSPD (Boehringer Mannheim) and recorded on X-ray film (Kodak XAR-5).

PCRs.

For generation of the 2.19-kb *celA* fragment oligonucleotide primers 5'-GAAGATCTAGTACTAGTATGGCTTCCCTACG

ATCC-3' (*cel578up*) and 5'-GAAGATCTAACAGGGTAGA AGCGGGAGG-3' (*cel2752low*) were used with Vent DNA polymerase (New England Biolabs, Beverly, MA). Since primers were designed with a *Bgl*III restriction site, the PCR amplificate could be cloned into the *Bam*HI site of vector pSPT19 (Roche Diagnostics, Mannheim, Germany) resulting in pSPT19:*celA*.

With primers pCRcel593 (5'-GGGAATTCAGTACTCCTT ATATGACATTTTCGCC-3') and pCRcel1860 (5'-GGAGATC TAAGCTTTCAGCCACTTCGCTGATACAG-3') the catalytic domain of *CelA* was amplified. The *EcoRI*/*Hind*III-digested amplificate (1.3 kb) was integrated into likewise digested pDM302, resulting in plasmid pHJ1200.

Primer extension.

The primer was a synthetic 32-mer oligonucleotide (5'-CG CAAGGAGGACGAGCCGAAGCACTAAGGATG-3') complementary to the 5' end of the *celA* gene. Annealing reactions, first-strand cDNA synthesis, and sequencing were carried out as reported by Dreier et al. (1997).

Plant growth conditions and virulence assay.

Plant growth conditions were according to Dreier et al. (1997). Virulence of *Cmm* and strains carrying hybrid plasmids was tested by infection of tomato plants (*Lycopersicon esculentum* cv. Moneymaker) as described previously (Dreier et al. 1997).

Recombinant DNA techniques.

Plasmid pCM1 was isolated as reported by Meletzus and Eichenlaub (1991). Vector DNA isolation from *E. coli* and

Table 2. Strains and plasmids used in this work

Strain or plasmid	Genotype or characteristic	Source/reference
Bacteria		
JM109	F' <i>traD36 lacI^q Δ(lacZ)M15 pro A⁺B⁺e14⁻(McrA⁻) Δ(lac-proAB) thi gyrA96 (Nal^r) endA1 recA1 hsdR17 (r_k⁻ m_k⁺)_{sup}E44 relA1 supE44</i>	Yanish-Perron et al. 1985
XL1-Blue	F':Tn10 <i>pro A⁺B⁺ lacI^q Δ(lacZ)M15/recA1 endA1 gyr96 (Nal^r) thi hsdR17 (r_k⁻ m_k⁺)_{sup}E44 relA1 lac</i>	Bullock et al. 1987
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> (<i>Cmm</i>) NCPPB382	Virulent on tomato, type strain	Meletzus and Eichenlaub 1991
<i>Cmm</i> CMM101	Plasmid-cured derivative of NCPPB382, lacking pCM2	Meletzus and Eichenlaub 1991
<i>Cmm</i> CMM100	Plasmid-free cured derivative of NCPPB382	Meletzus and Eichenlaub 1991
<i>Cmm</i> CMM71-S	Streptomycin resistant <i>Cmm</i> isolate	Dreier et al. 1997
<i>Cmm</i> CMM-H4	CMM101 (pCM1-B1:: <i>aacC1</i>)	This work
<i>Cmm</i> CMM-H4c	CMM-H4 (pDM302:B1)	This work
Plasmids		
pDM302	<i>Escherichia coli</i> - <i>Clavibacter</i> sp. shuttle vector Cm ^r , Neo ^r	Meletzus et al. 1993
pDM302:B1	Nm ^r , pDM302: <i>Bgl</i> II-1	This work
pHJ-1	Ap ^r , pSVB30: <i>Bgl</i> II-1	This work
pHJ10	Ap ^r , pHJ-1: <i>Bgl</i> II-1-Δ <i>Clal</i> / <i>Xba</i> I (positions 2816–3196)	This work
pHJ1010	Nm ^r , pDM302: pHJ10 <i>EcoRI</i> / <i>Hind</i> III-insert	This work
pHJ1102	Nm ^r , pDM302: <i>Bgl</i> II-1-Δ <i>Sph</i> I (positions 1984–2858)	This work
pHJ1103	Nm ^r , pDM302: <i>Bgl</i> II-1-Δ <i>Nco</i> I (positions 2374–2928)	This work
pHJ1104	Nm ^r , pDM302: <i>Bgl</i> II-1 (positions 1–1626)	This work
pHJ1105	Nm ^r , pDM302: <i>Bgl</i> II-1 (positions 1626–3197)	This work
pHJ1107	Nm ^r , pDM302: <i>Bgl</i> II-1 (positions 716–3112)	This work
pHJ1200	Nm ^r , pDM302:CMCase (positions 592–1857)	This work
pHJ-III	Ap ^r , pHJ10: <i>Bgl</i> II-1 (<i>Bsp</i> 120I ₇₁₆ :: <i>aacC1</i>)	This work
pSPT19	Ap ^r , pUC derivative	Boehringer, Mannheim, Germany
pSPT19: <i>celA</i>	Ap ^r , pSPT19: 2.19-kb internal <i>celA</i> polymerase chain reaction fragment	This work
pSVB30	Ap ^r , pUC derivative	Arnold and Pühler 1988

Clavibacter spp. and electroporation of *Cmm* were done according to Dreier et al. (1997). Plasmid DNA was isolated from *E. coli* following the Qiagen plasmid isolation kit protocol (Qiagen, Hilden, Germany). Plasmid constructions were carried out by standard procedures (Sambrook et al. 1989).

Southern hybridization.

The 2.19-kb *celA*-specific probe was obtained by *EcoRI*/*HindIII* double digestion of plasmid pSPT19:*celA*, followed by JETSORB (Genomed, Bad Oeynhausen, Germany) agarose gel purification and subsequent random priming according to the DIG DNA Labeling Kit manual (Boehringer Mannheim). A 1.6-kb, JETSORB-purified *SmaI* fragment of pHN5 was labeled likewise, to obtain an *aacC1*-specific probe.

Total DNA was prepared from 5 ml of late log-phase cultures as described by Altenbuchner and Cullum (1984). Total DNA and plasmid DNA were cleaved with *Bgl*III, and DNA fragments were separated by agarose gel electrophoresis (Southern 1979). Transfer to Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and detection were performed as described by Dreier et al. (1997).

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