Characterization of the Plasmid Encoded Virulence Region *pat-1* of Phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*

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The tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382, causing bacterial wilt and canker, harbors two plasmids, pCM1 (27.5 kb) and pCM2 (72 kb), carrying genes involved in virulence. The region of plasmid pCM2 encoding the pathogenicity locus *pat-1* was mapped by deletion analysis and complementation studies to a 1.5-kb *Bgl*III/*Sma*I DNA fragment. Introduction of the *pat-1* region into endophytic, plasmid-free isolates of *C. michiganensis* subsp. *michiganensis* converted these bacteria into virulent pathogens. Based on the nucleotide sequence of the *pat-1* region, an open reading frame (ORF1) can be predicted, coding for a protein of 280 amino acids and 29.7 kDa with homology to serine proteases. Introduction of a frame-shift mutation in ORF1 leads to a loss of the pathogenic phenotype. Northern (RNA) hybridizations identified an 1.5-knt transcript of the *pat-1* structural gene. The site of transcription initiation was mapped by primer extension and a typical –10/–35 region was located with significant homology to the consensus *Escherichia coli* σ^{70} and *Bacillus subtilis* σ^{43} promoters. Downstream of the *pat-1* structural gene, a peculiar repetitive sequence motif (*pat-Irep*) is located, consisting of 20 direct tandem repeats preceded by a run of 14 guanosine residues. DNA sequences homologous to *pat-Irep* were isolated and characterized from four virulent *C. michiganensis* subsp. *michiganensis* strains exhibiting a high extent of structural conservation. The deletion of this repetitive sequence reduced virulence significantly but did not lead to a complete loss of the virulence phenotype.

Additional keyword: gram-positive bacteria.

The gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* causes bacterial canker of tomato (*Lycopersicon esculentum* Mill.) (Davis et al. 1984; Rothwell 1968). This pathogen is a target of international quarantine regulations (Kahn 1982; Franken et al. 1993) since bacterial

canker of tomato has caused major economic losses of up to 60% in commercial tomato production worldwide (Strider 1969), and neither resistant tomato cultivars nor effective chemical controls of this pathogen are available (Thompson 1986). Generally, *C. michiganensis* subsp. *michiganensis* is transmitted by contaminated seeds or transplants (Fatmi and Schaad 1988; Gitaitis et al. 1991), and therefore the spreading of this serious pathogen can only be controlled by detection and elimination of infected plant tissue. The bacteria infect the host plant via wounds and invade the xylem vessels; systemic infection of the host follows. To explain the mechanisms for the induction of bacterial wilt several hypotheses have been presented. Exopolysaccharides (EPS) and glycoproteins produced by the bacteria were inferred to hinder water transport by plugging the xylem vessels in an in vitro assay on tomato seedlings (Rai and Strobel 1968a, 1968b; van den Bulk et al. 1989). However, analysis of the EPS of various *C. michiganensis* subsp. *michiganensis* strains differing in virulence and pathogenic phenotype of EPS-negative mutants indicated that EPS is not a crucial pathogenicity factor in *C. michiganensis* subsp. *michiganensis* (Bernpohl et al., in press).

It was also suggested that wilting may result from an enzymatic attack on xylem vessels and adjacent parenchymatic cells (Wallis 1977; Benhamou 1991). Work from several laboratories has claimed that extracellular cell-wall-degrading enzymes have an important role in disease development. Production of exoenzymes such as endocellulase (Meletzus et al. 1993), polygalacturonase (Beimen et al. 1992), pectinmethylesterase (Strider 1969), and xylanase (Beimen et al. 1992) were identified in vitro and in vivo in *C. michiganensis* subsp. *michiganensis*. It is tempting to speculate that the macerating activity of the exoenzymes on plant cells is the reason for wilt development. Furthermore, a combined action with other virulence genes active on cell wall components and/or genes involved in other mechanisms of pathogenic action is still possible.

For a genetic approach to the mechanisms of pathogenicity, vectors and transformation systems are available for *C. michiganensis* subsp. *michiganensis* (Meletzus and Eichenlaub 1991; Laine et al. 1996). The involvement of plasmids pCM1 and pCM2 in pathogenicity along with the localization of an endocellulase on plasmid pCM1 was reported recently (Meletzus et al. 1993). In the present report, we describe the molecular characterization of the pathogenicity locus *pat-1* of plasmid pCM2 (Meletzus et al. 1993), which is directly involved in the development of disease symptoms.

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Nucleotide sequence data are to be found at GenBank as accession numbers L78159, L78160, L78161, and L78162.

RESULTS

Physical mapping of the pathogenicity region *pat-1*.

The involvement of plasmid pCM2 in pathogenicity along with the induction of wilting symptoms in infected tomato plants was reported recently (Meletzus et al. 1993). The virulence region *pat-1* of plasmid pCM2 was isolated as the 3.7-kb *Bgl*III-7 restriction fragment inserted into the *E. coli*-*Clavibacter* shuttle vector pDM306, resulting in the hybrid plasmid pJD2107. Since the hybrid *Clavibacter* strains CMM100 (pJD2107 α) and CMM100(pJD2107 β) induced disease symptoms after the same incubation time and to the same extent, expression of *pat-1* was orientation-independent, indicating that the *pat-1* promoter as well as the complete coding sequence were present on the 3.7-kb DNA fragment. To identify the corresponding virulence gene and its promoter on this DNA fragment, overlapping restriction fragments and *Exo*III/S1-generated deletion products were subcloned into *E. coli*-*Clavibacter* shuttle vectors pDM302 and pDM306 (Fig. 1). The hybrid plasmids carrying DNA fragments of pCM2 were isolated from *E. coli* and then used to transform the plasmid-free strain *Clavibacter* CMM100. After verification of the plasmid content by DNA reisolation, the transformed derivatives of CMM100 were subsequently tested for their ability to induce the specific disease symptoms, i.e., leaf wilting or biomass reduction after infection of *L. esculentum* cv. Monenheim. The results of such virulence assays are presented in Figure 1 and led to the identification of an 1.5-kb *Bgl*III-*Sma*I region carrying the *pat-1* locus required for the induction of disease symptoms (Fig. 1).

pat-1 enhances the virulent phenotype in low-virulence *C. michiganensis* subsp. *michiganensis* strains.

The virulence of several isolates of *C. michiganensis* subsp. *michiganensis* of different geographical origin was evaluated in plant assays (Table 1) and a classification by virulence was

made after determination of the wilting index, biomass production, and colonization of infected tomato plants. *C. michiganensis* subsp. *michiganensis* strains were grouped into three virulence classes: virulent, low virulence, and avirulent strains. Virulent isolates colonized the host plant effectively with bacterial titers higher than 10⁷ CFU per g of plant material, induced typical wilting symptoms, and led to a significant biomass reduction of infected plants. In comparison, low-virulence *C. michiganensis* subsp. *michiganensis* strains were not capable of inducing wilting symptoms even 8 weeks after infection, although they colonized the plant to the same extent as virulent strains. However, a slight reduction in biomass of plants infected with low-virulence isolates was detected. With respect to colonization and virulence, low-virulence strains have the same phenotype as the plasmid-free derivative of NCPPB382, strain CMM100. In addition, no plasmids were detected in these strains (data not shown). Avirulent strains NCPPB3123, NCPPB3285, NCPPB3286, and ATCC4450 did not colonize tomato plants and caused no disease symptoms. The bacterial titer in plants 3 weeks after infection did not increase but stayed at the level of inoculation (<10⁴ CFU/ml). Transformation of low-virulence *C. michiganensis* subsp. *michiganensis* strains with plasmid pJD2107 carrying the *pat-1* locus leads to a conversion to the wilt-inducing phenotype, whereas no restoration of virulence was observed in avirulent isolates (Table 1). For strains NCPPB3285, NCPPB3286, NCPPB 1574, and ATCC10202 we found a virulence phenotype different from that given in the strain collection (compare Tables 1 and 2), which may be due to different virulence assays.

The nucleotide sequence of *pat-1*.

In order to obtain sequence information on the pathogenicity-related gene *pat-1*, the *Bgl*III-7 restriction fragment of pCM2 was inserted in both orientations into the single *Bam*HI restriction site of the *E. coli* sequencing vector pSVB30 (Arnold and Pühler 1988). The nucleotide sequence of the in-

Fig. 1. Functional testing of pJD2107 deletion and frame shift mutation derivatives in plasmidless strain CMM100. Remaining part of cloned DNA present in each plasmid is shown by a horizontal bar. Minimal region encoding *pat-1* structural gene (ORF1) is marked on linear map by an arrow. Both orientations of the 3.7-kb insert (pJD2107 α / β) were tested and found to restore virulence in strain CMM100 to the same extent. Exact endpoints of deletion plasmids are shown with reference to nucleotide position (see Figure 2). Frame shift mutation (+1) is marked as a triangle at nucleotide position 766. Virulence of strains is estimated by the wilting index, number of days when 50% of infected plants (n = 30) show wilting symptoms, and biomass (dry weight) of infected tomato plants 4 weeks after inoculation, compared with control plants. Values for standard deviation are given.

ternal *Bgl*III-*Sma*I fragment carrying the virulence region was determined on both strands (Fig. 2). Computer-based analysis of the sequence revealed a single open reading frame (ORF1), from ATG₁₈₀ to the termination codon TGA₁₀₂₀. The deduced protein consists of 280 amino acids (aa) with a calculated molecular mass of 29.7 kDa. In order to verify the involvement of the putative ORF1 gene product in the development of disease symptoms, a frame shift mutation (+1) was introduced at nucleotide position 766 (Fig. 1). The corresponding hybrid plasmid pJD2107E has a 4-base pair (bp) insertion at the *Eco*RI site of pJD2107, constructed by filling in the cohesive ends with Klenow polymerase, followed by religation, thus generating a TAA₇₈₀ stop codon and a putative, truncated ORF1 protein of 21.1 kDa. The corresponding strain CMM100(pJD2107E) was found to be unable to induce disease symptoms in infection tests (Fig. 1), indicating that ORF1 is the pathogenicity locus of the *pat*-1 region.

A putative ribosomal binding site is located 5 bp upstream of start codon ATG₁₈₀ (5'-GGGCGC-3'; underlined in Figure 2). The first 33 aa residues of the N terminus of the ORF1 protein resemble a typical signal sequence (von Heijne 1986) with two positively charged amino acids (Arg₆ and Arg₉) followed by 21 hydrophobic amino acids and a polar region. A putative cleavage site of the signal peptide is located between the alanine and valine residues at positions 33 and 34 of the amino acid sequence of ORF1 (Fig. 2). Processing would result in a mature ORF1 protein with a molecular mass of 26.2 kDa. The presence of a signal peptide suggests that ORF1 may be secreted via the *sec*-dependent pathway (Salmond 1994).

A computer search for possible homologies between *C. michiganensis* subsp. *michiganensis* ORF1 and other proteins indicated significant homologies to serine proteases from *Lysobacter enzymogenes* (Epstein and Wensink 1988), *Streptomyces griseus* (Henderson et al. 1987), and a *Staphylococcus* sp. (GenBank accession number S25140). Among these, the alpha-lytic protease of *Lysobacter enzymogenes* (α -LP) shared 28.5% identical and 65% homologous amino acid residues over a region of 102 aa with ORF1. Furthermore, at amino acid position 230 of the ORF1 gene product the consensus motif of the trypsin family of serine proteases (G-D-S-G-G) with serine at the active site (Brenner 1988) was identified (Fig. 3), while the second sequence motif ([LIVM]-[ST]-A-[STAG]-H-C) with histidine at the active site characteristic for this serine protease family was missing.

Downstream of the *pat*-1 structural gene ORF1 a peculiar sequence motif is located, consisting of 20 direct tandem repeats of 7 to 8 bp preceded by a run of 14 guanosine residues (Figs. 2 and 4), termed *pat*-1rep. In addition, an inverted repeat, IR4, which may function as a rho-independent transcriptional terminator, is located close to the last 7 bp repeat (Fig. 2).

Analysis of *pat*-1 transcripts.

To investigate the expression of the *pat*-1 region, the length of the *pat*-1 mRNA was determined by Northern (RNA) hybridization with an internal DNA fragment of ORF1 as probe. The total RNA was analyzed from cells of CMM100 harboring one of the following plasmids: pDM306, pJD2107 α , or pJD2107 β (Fig. 5). A single transcript, which migrates as an RNA molecule of approximately 1,500 nt, was detected in CMM100 strains harboring pJD2107 α and pJD2107 β . No such RNA molecule was observed in the controls, the plas-

mid-free strain CMM100 or CMM100(pDM306). Since plasmids pJD2107 α and pJD2107 β carry the *pat*-1 region in different orientations, transcription of the *pat*-1 region seems to occur from the native *pat*-1 promoter.

The 5' end of the *pat*-1 mRNA was mapped by the primer extension method (Fig. 6). The start of transcription was located 97 nt upstream from the start codon of ORF1 (G at nucleotide sequence position 78). Upstream of the transcription initiation site, a typical -10/-35 region with significant homology to the *E. coli* σ^{70} consensus promoter (Rosenberg and Court 1979) was found (Fig. 2). In addition, there are four sequence repeats (DR1 to DR4) in the promoter region that may function as regulatory signals (Fig. 2). The mapping of the initiation site of the *pat*-1 transcript, along with its size, indicates that termination must occur downstream of the repetitive sequence *pat*-1rep. According to computer analysis, the 3' end of the *pat*-1 mRNA is able to form a highly stable fold-back structure, consisting of a stem-loop and a terminal rho-independent like terminator (Fig. 7).

The repetitive DNA region *pat*-1rep, a common motif in virulent *C. michiganensis* subsp. *michiganensis* strains.

By Southern blot analysis it was found that only virulent strains of *C. michiganensis* subsp. *michiganensis* carry DNA

Table 1. Reconstitution of virulence

Strain	Colonization (CFU/g plant matl.) ^a	Wilt induc- tion ^b	Bio- mass (%) ^c	Aggressive- ness ^d
NCPPB2979	5.2×10^8	+ (7)	62	Virulent
NCPPB3120	2×10^8	+ (7)	58	Virulent
NCPPB3121	3.1×10^8	+ (6)	50	Virulent
NCPPB3223	6.4×10^8	+ (7)	56	Virulent
NCPPB3264	6.1×10^8	+ (7)	58	Virulent
CMM100	9.6×10^8	—	75	Low virulence
CMM100 (pDM306)	6.1×10^8	—	76	Low virulence
CMM100 (pJD2107)	7.3×10^8	+ (12)	56	Virulent
NCPPB1574	8.4×10^7	—	75	Low virulence
NCPPB1574 (pDM306)	7.5×10^7	—	76	Low virulence
NCPPB1574 (pJD2107)	8.6×10^7	+ (12)	58	Virulent
ATCC10202	1.2×10^7	—	91	Low virulence
ATCC10202 (pDM306)	2.1×10^7	—	94	Low virulence
ATCC10202 (pJD2107)	1.8×10^7	+ (16)	81	Virulent
CMM71-S	2.4×10^7	—	94	Low virulence
CMM71-S (pDM306)	5.3×10^7	—	90	Low virulence
CMM71-S (pJD2107)	3.6×10^7	+ (16)	79	Virulent
NCPPB3285	5.3×10^7	—	100	Avirulent
NCPPB3285 (pDM306)	$<10^4$	—	96	Avirulent
NCPPB3285 (pJD2107)	$<10^4$	—	NT ^e	Avirulent
NCPPB3286	1.1×10^4	—	NT	Avirulent
NCPPB3286 (pDM306)	$<10^4$	—	NT	Avirulent
NCPPB3286 (pJD2107)	$<10^4$	—	NT	Avirulent
NCPPB3123	1.2×10^4	—	100	Avirulent
NCPPB3123 (pDM306)	$<10^4$	—	NT	Avirulent
NCPPB3123 (pJD2107)	$<10^4$	—	NT	Avirulent
ATCC4450	1.2×10^3	—	100	Avirulent
ATCC4450	$<10^4$	—	NT	Avirulent
ATCC4450 (pJD2107)	$<10^4$	—	NT	Avirulent

^a Bacterial titers determined on C medium 3 weeks after infection.

^b Values in parenthesis represent wilting index, number of days when 50% of infected plants show wilting symptoms.

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

^d Virulence classification: virulent (wilt induction, $>10^7$ CFU/g), low virulence ($>10^7$ CFU/g), avirulent strains ($<10^7$ CFU/g).

^e Not tested.

motifs homologous to *pat-Irep*, whereas such homologies were not detected in avirulent isolates (Dreier et al. 1995). In order to analyze the dissemination of the repetitive sequence motif *pat-Irep*, an amplification of the gene locus from the three *C. michiganensis* subsp. *michiganensis* strains NCPPB3120, NCPPB3121, and NCPPB3264 was carried out by polymerase chain reaction (PCR) with the oligonucleotide primers P1-rep and P3-rep. The amplified DNA fragments containing the repetitive sequence motif and varying in length between 162 and 219 bp were inserted into vector pSVB30

(Arnold and Pühler 1988) by using the restriction enzyme recognition sites *EcoRI/ScaI* (P1-rep) and *HindIII/ScaI* (P3-rep) provided by the PCR primers and cloned in *E. coli* JM109. DNA sequence analysis revealed a high degree of conservation of *pat-Irep* among different strains of *C. michiganensis* subsp. *michiganensis*. In all strains examined a polyG-motif consisting of eight (NCPPB3264) to 14 guanosine residues (NCPPB382) is followed by several direct repeats with predominant repeat DR2 and an inverted repeat IR4 (Fig. 4). Computer-based analysis of possible secondary structures of

Table 2. Strains and plasmids used in this work

Strain or plasmid	Genotype or characteristic (virulence according to strain collections)	Source or reference
Strains		
<i>Escherichia coli</i> JM109	F ⁺ <i>traD36 lacI^q</i> , Δ (<i>lacZ</i>) M15, <i>proAB/recA1, recA1, endA1, gyrA96</i> (Nal ^r) <i>thi, hsdRI7</i> ($r_k^-m_k^+$), <i>supE44, relA1, \Delta(<i>lac-proAB</i>)</i>	Yanisch-Perron et al. 1985
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> CMM100	Plasmid-free cured derivative of NCPPB382	Meletzus and Eichenlaub 1991
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB1574	Virulent on tomato (<i>L. esculentum</i>), Z. Klement et al., Hungary, 1963	National Collection of Plant Pathogenic Bacteria, Hatching Green, Harpenden, England (NCPPB)
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB2979	Virulent on tomato (<i>L. esculentum</i>), type strain, Z. Klement et al., Hungary, 1957	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3120	Virulent on tomato (<i>L. esculentum</i>), J. Donaldson, UK, 1979	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3121	Virulent on tomato (<i>L. esculentum</i>), J. Donaldson, UK, 1979	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3123	Avirulent on tomato (<i>L. esculentum</i>), E. Echandi, USA, 1972	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3223	Virulent on tomato (<i>L. esculentum</i>), M. Fenton, UK, 1981	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3264	Virulent on tomato (<i>L. esculentum</i>)	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3285	Low virulence on tomato (<i>L. esculentum</i>), D. E. Stead, UK, 1983	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB3286	Low virulence on tomato (<i>L. esculentum</i>), D. E. Stead, UK, 1983	NCPPB
<i>C. michiganensis</i> subsp. <i>michiganensis</i> CMM71-s	streptomycin resistant isolate, E. Giesbach, Germany, 1994	E. Griesbach, <i>unpublished</i>
<i>C. michiganensis</i> subsp. <i>michiganensis</i> ATCC10202	Virulent on tomato (<i>L. esculentum</i>), P. A. Ark, U.S.	ATCC
<i>C. michiganensis</i> subsp. <i>michiganensis</i> ATCC4450	R. E. Buchanan, U.S.	ATCC
Plasmids		
pSVB30	Ap ^r , pUC derivative, sequencing vector	Arnold and Pühler 1988
pDM302	Cm ^r , Neo ^r , <i>E. coli</i> - <i>Clavibacter</i> sp. shuttle vector	Meletzus et al. 1993
pDM306	Gn ^r , Neo ^r , <i>E. coli</i> - <i>Clavibacter</i> sp. shuttle vector	Meletzus et al. 1993
pJD1572	Neo ^r , pDM302: 1.5-kb <i>HindIII/SmaI</i> <i>pat-1</i> DNA fragment (<i>pat-1</i> pos. 1 to 1513 nt)	This work
pJD1671	Neo ^r , pDM302: <i>ExoIII/S1</i> deletion <i>BglII-7</i> (<i>pat-1</i> pos. 1 to 1258)	This work
pJD1672	Neo ^r , pDM302: pCM2 <i>BglII-7</i> Δ <i>EcoRI/HindIII</i> (<i>pat-1</i> pos. 1 to 766)	This work
pJD1676	Neo ^r , pDM302: <i>ExoIII/S1</i> deletion <i>BglII-7</i> (<i>pat-1</i> pos. 766 to 3148 nt)	This work
pJD2107 α	Neo ^r , pDM306: pCM2 3.7-kb <i>BglII-7</i> fragment, <i>pat-1</i> , α orientation	Meletzus et al. 1993
pJD2107 β	Neo ^r , pDM306: pCM2 3.7-kb <i>BglII-7</i> fragment, <i>pat-1</i> , β orientation	This work
pJD2107E	Neo ^r , pJD2107 α +1 mutation at <i>pat-1</i> pos. 766 nt	This work
pJD2771	Neo ^r , pDM306: 3.4-kb <i>BamHI/BglII</i> <i>pat-1</i> DNA fragment (<i>pat-1</i> pos. 406 to 3756 nt)	This work
pJD2772	Neo ^r , pDM306: pCM2 <i>BglII-7</i> Δ <i>BamHI/HindIII</i> (<i>pat-1</i> pos. 406 to 3756 nt)	This work
pJD2773	Neo ^r , pDM302: 1.1-kb <i>BamHI/SmaI</i> <i>pat-1</i> DNA fragment (<i>pat-1</i> pos. 406 to 1513 nt)	This work
pSVB382rep	Ap ^r , pSVB30: 219-bp polymerase chain reaction (PCR) product of repetitive DNA <i>pat-1rep</i> of NCPPB382 (<i>pat-1</i> pos. 1358 to 1577 nt)	This work
pSVB3120rep	Ap ^r , pSVB30: 183-bp PCR product of repetitive DNA of NCPPB3120	This work
pSVB3121rep	Ap ^r , pSVB30: 178-bp PCR product of repetitive DNA of NCPPB3121	This work
pSVB3264rep	Ap ^r , pSVB30: 162-bp PCR product of repetitive DNA of NCPPB3264	This work
pSVB:B7 α	Ap ^r , pSVB30: pCM2 3.7-kb <i>BglII-7</i> fragment, <i>pat-1</i> , α orientation	This work
pSVB:B7 β	Ap ^r , pSVB30: pCM2 3.7-kb <i>BglII-7</i> fragment, <i>pat-1</i> , β orientation	This work

these regions show a high extent of thermodynamically stable, double-stranded RNA, with a stem-loop structure at the 3' end formed by inverted repeat IR4 (Fig. 7).

It is interesting to note that deletion of 255 bp covering the complete *pat1-rep* region in plasmid pJD1671 (Fig. 1) resulted in reduced virulence of strain CMM100(pJD1671). The induction of wilting symptoms was delayed and the gain of biomass of infected plants was higher, compared with strains harboring plasmids carrying the complete *pat-1* locus.

DISCUSSION

In this study we analyzed the pathogenicity locus *pat-1* of *C. michiganensis* subsp. *michiganensis* responsible for the pathogenic phenotype mediated by plasmid pCM2 of strain NCPPB382. Deletion analysis mapped the region essential for wilt induction to a 1.5-kb *Bgl*III/*Sma*I DNA fragment containing ORF1 and the repetitive sequence motif *pat-1rep*.

A transcript of about 1.5-knt of the *pat-1* structural gene was identified by Northern blot analysis in *C. michiganensis* subsp. *michiganensis* transformants carrying a *pat-1* expressing plasmid. The transcription initiation site was mapped by primer-extension experiments at 78 nt. Analysis of the nucleotide sequence upstream of this start point revealed similarities to the -10/-35 region of the *E. coli* σ^{70} consensus promoter (Rosenberg and Court 1979). A comparison of the *pat-1* promoter from *C. michiganensis* subsp. *michiganensis* with characterized promoters of other GC-rich, gram-positive bacteria, and especially with the three promoter sequences determined from *Clavibacter xyli* subsp. *cynodontis* (Haapalainen et al. 1996), did not reveal homologies sufficient for the definition of a *Clavibacter*-specific promoter consensus sequence. This dilemma is also encountered in other coryneform bacteria and the streptomycetes and may only be solved when more promoter sequences have been determined. Several direct repeats of 6 to 8 nt were found on both sides of the transcription start site. These may represent binding sites for specific regulatory proteins that modulate expression of the *pat-1* gene. Isolation and characterization of other *C. michiganensis* subsp. *michiganensis* promoters are in progress to obtain more information about the structure and function of elements involved in the initiation of transcription.

In *E. coli* and *B. subtilis*, ribosome binding sites are characterized by complementarity to a region near the 3' end of 16S rRNA (Shine and Dalgarno 1974; Gold et al. 1981). The sequence of the 3' end of 16S rRNA of *C. michiganensis* subsp. *michiganensis* has not been determined yet. Despite the absence of a typical ribosome binding site for the *pat-1* structural gene, a sequence motif (5'-CGGGCG-3') was found in both the *pat-1* and *celA* genes, located 5 to 6 nt upstream of the translation start codon (D. Meletzus, *unpublished*), which may represent the translation initiation site for *C. michiganensis* subsp. *michiganensis*.

Since a frame shift mutation in ORF1 leads to a loss of the pathogenic phenotype, this suggests that translation of ORF1 is required and that this gene product is a pathogenicity determinant of plasmid pCM2. The hydropathic profile indicates that the ORF1 protein may be secreted via signal peptide mediated transport. Many bacterial plant pathogens produce extracellular products, such as cell-wall-degrading enzymes, toxins, DNA, hormones, siderophores, and signaling molecules, that contribute to virulence (for a recent review see Collmer and Bauer 1994). Among these, cellulases, pectinases, and proteases were discussed as important virulence factors of many plant pathogens (reviewed by Daniels et al. 1988). By computer homology search we found significant homologies to serine proteases from *Lysobacter enzymogenes* (Epstein and Wensink 1988), *Streptomyces griseus* (Henderson et al. 1987), and a *Staphylococcus* sp. (GenBank accession number S25140). The homologous regions are part of the catalytic site and therefore functionally important. Besides the serine active site (domain C), most serine proteases belonging to the trypsin family also have the histidine active site (domain A) (Brenner 1988; Kraut 1977). On the other hand, as is the case for the ORF1 protein, some serine proteases, such as the human complement components C1r and C1s (Kusomoto et al. 1988), are lacking the serine protease domain A. The involvement of proteases in pathogenesis was discussed for *Xanthomonas campestris* pv. *campestris* (Dow et al. 1990), *Pseudomonas syringae* pv. *tomato* (Bashan et al. 1986), and *Erwinia chrysanthemi* (Wandersman et al. 1987). Multiple protease activities have been found in most of these bacteria, although their significance for pathogenesis is not completely understood. In some cases, the role of bacterial

ORF1	160	G A R N R S G Q E S S V Q V A G T K V P A D R E I F C T S G A I T
α -LP	275	L L P R V A N G S S F V T V R G S T E A A V G A A V C R S G R T T
ORF1	193	G I L C N W V S A P P P R G - L E I G S H Q V V A E T F S A A T R
α -LP	308	G Y Q C G T I T A K N V T A N Y A E G A V R G L T Q G N A C M G R
ORF1	225	Q G D S G G P V V S R D M K I I G V I C D G G L P G S G D D T Y M
α -LP	341	- G D S G G S W I T S A G Q A Q G V M S G G N V Q S N G N N C G I
ORF1	258	S Y L P I S V L F - R E Q P Y Y
α -LP	373	P A S Q R S S L F E R L Q P I L

Fig. 3. Alignment of deduced amino acids 160 to 272 of *Clavibacter michiganensis* subsp. *michiganensis* ORF1 and *Lysobacter enzymogenes* α -LP (Epstein and Wensick 1988). Identical amino acids indicated by gray shading and conserved exchanges are boxed. Region carrying serine protease motif C of trypsin family indicated against black background.

gene product. This may be explained by a narrow substrate specificity or great instability of the gene product. In *X. campestris* pv. *armoraciae*, bacterial protease production can only be detected in planta at bacterial titers of about 10^8 CFU/ml (Dow et al. 1993). In the *Clavibacter*-tomato interaction,

wilting symptoms occur at a late stage of infection, after the bacterial population has reached titers of about 10^9 CFU per g of plant tissue (Meletzus et al. 1993). The expression of ORF1 could therefore be regulated by plant factors. Further experiments will be necessary to study the expression and mode of action of the ORF1 gene product in planta.

Deletion of the peculiar repetitive sequence *pat-1rep* reduced virulence as observed with CMM100(pDJ1671). The induction of wilting symptoms was delayed and the biomass of infected plants was higher, compared with strains harboring plasmids carrying the complete *pat-1* locus. Therefore, this sequence motif seems to have an effect on the virulence phenotype. Computer-aided analysis of secondary structure of the *pat-1* mRNA showed that a complex fold-back structure can be formed in the 3'-terminal region. Besides its function as a terminator for transcription, there is the possibility that the 3'-terminal secondary structure may protect the *pat-1* transcript against degradation by cellular endoribonucleases and 3' exoribonucleases. RNaseII and polynucleotide phosphorylase, the major bacterial exonucleases involved in mRNA turnover, rapidly degrade single-stranded RNA from the 3' end, but are impeded by 3' stem-loop structures (for review see Belasco and Brawerman 1993). If this occurs in *Clavibacter* then deletion of the *pat-1rep* region may result in a higher turnover of the *pat-1* mRNA, less *pat-1* gene product, and reduced virulence, as observed with strain CMM100(pJD1671). Experiments are in progress to determine whether the repeats of the *pat-1rep* region indeed contribute to mRNA stability.

The inverted repeat at the end of the *pat-1rep* region (IR4) can form a highly stable hairpin-loop structure (-38.3 kcal/mol) and may represent a transcription termination signal analogous to those observed in *E. coli* (Rosenberg and Court 1979). Like rho-independent terminators found in *E. coli*, this inverted repeat is followed by a downstream run of uridine residues.

Fig. 5. Northern (RNA) hybridization analysis of *Clavibacter michiganensis* subsp. *michiganensis* *pat-1* region. RNA extracted from strain CMM100 (lane 1), CMM100(pDM306) (lane 2), CMM100(pJD2107 α) (lane 3), and CMM100(pJD2107 β) (lane 4) grown in C medium. Denatured total RNA subjected to formaldehyde agarose gel electrophoresis, blotted to a nylon membrane, and hybridized to a [α - 32 P]dATP-labeled polymerase chain reaction fragment of the ORF1 gene (0.6 kb). Approximate size of *pat-1*-specific transcript determined against an RNA molecular size ladder (lane M).

Recently it was found that the *pat-1rep* region was always associated with *pat-1* and virulent isolates of *C. michiganensis* subsp. *michiganensis* and located on plasmids as shown by Southern hybridization and PCR (Dreier et al. 1995). The characterization of *pat-1rep* homologous DNA sequences obtained from other *C. michiganensis* subsp. *michiganensis* strains demonstrated a high extent of structural conservation of this region. The loss of plasmids is correlated with a reduction of virulence (Vidaver 1982), as shown for strain NCPPB382 by curing experiments (Meletzus et al. 1993). As reported recently, the virulent phenotype was restored in CMM100 due to independent introduction of the two plasmid-encoded virulence factors (Meletzus et al. 1993).

Now that a vector derived from pCM2 is available (Laine et al. 1996) it can be tested whether simultaneous introduction of both pathogenicity loci will restore virulence to the level of the parental strain NCPPB382. In addition, we were able to convert endophytic, plasmid-free isolates of *C. michiganensis* subsp. *michiganensis* to virulent pathogens by the same strategy. Cloning of *pat-1* in avirulent strains did not enhance aggressiveness, because bacterial propagation in planta failed. These results give additional evidence to support the hypothesis that genes involved in wilt induction in *C. michiganensis* subsp. *michiganensis* are a plasmid-borne trait.

In conclusion, our data demonstrate the important role of the *pat-1* gene product in pathogenicity. The current objective of our work is to determine the function of this virulence factor. This will possibly help us to understand more about the interaction between *C. michiganensis* subsp. *michiganensis* and its host plant.

Fig. 6. Determination of 5' end of *pat-1* mRNA by primer extension. Experiment carried out with total RNA isolated from strain CMM100 (pJD2107). Length of extended DNA products determined on a urea-polyacrylamide gel in parallel with sequencing ladders, with same primer as used for primer extension assay. Sequence of region is shown on left. Transcriptional initiation site indicated by an arrow.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 2. Growth conditions were as described previously (Meletzus and Eichenlaub 1991). For reisolation of bacteria from infected plants, a modified SCM medium (Beimen et al. 1992; Meletzus et al. 1993) was used. *Clavibacter* and *E. coli* strains harboring plasmids were grown on selective LB medium as described previously (Meletzus and Eichenlaub 1991).

Recombinant DNA techniques.

For plasmid isolation, *Clavibacter* cells were treated with lysozyme (10 mg/ml) in isotonic buffer HBI containing 50 mM glucose, 10 mM trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA), 25 mM Tris-HCl, pH 8.0 for 1 h at 37°C. Purification of plasmid DNA from *E. coli* and *Clavibacter michiganensis* subsp. *michiganensis* was carried out with QIAGEN Mini and Midi Plasmid kits (Qiagen, Hilden, Germany). Preparation of high molecular mass, native plasmids of *C. michiganensis* subsp. *michiganensis* was performed as previously described (Meletzus and Eichenlaub 1991). Transformation of *E. coli* was done by a method described by Sambrook et al. (1989). To obtain *C. michiganensis* subsp. *michiganensis* cells for electroporation, bacteria were grown to late-log-phase in 100 ml of TYN medium (10 g of tryptone, 2.5 g of yeast extract, 5 g of NaCl per liter, pH 7.4) at 25°C (about 10^9 cells per ml). Cells were harvested by centrifugation at $3,000 \times g$ for 10 min at 4°C and washed in an equal volume of double-distilled H₂O. Bacteria were resuspended in 10 ml of 10% (vol/vol) glycerol followed by the addition of 100 µl of lysozyme solution (4 mg/ml). Lysozyme treatment was performed for 20 min at 28°C. Cells were then washed twice in 10% glycerol and finally resuspended in 1 to 2 ml of 10% glycerol. Aliquots of 200 µl of lysozyme-treated cells were used for electroporation as described previously (Meletzus and Eichenlaub 1991). After applying the desired pulse, cells were washed immediately from the cuvette with 200 µl of osmotically protective SB medium (10 g of tryptone, 5 g of yeast extract, 4 g of NaCl per 500 ml, pH 7.2, 460 ml of 1 M sorbitol, 20 ml of 1 M CaCl₂, 20 ml of 1 M MgCl₂) (Yoshihama et al. 1985), and incubated for 2 h at 25°C before plating onto SB agar plates containing the appropriate antibiotics.

Plasmid DNA constructions and analysis were carried out by standard procedures (Sambrook et al. 1989). Restriction enzymes, DNA modifying enzymes, and T4 DNA ligase were obtained from New England Biolabs (Schwalbach/Taunus, Germany), Boehringer Mannheim (Mannheim, Germany), and Gibco/BRL (Berlin), respectively. The construction of subclones of the 3.7-kb DNA fragment of plasmid pCM2 is summarized in Figure 1. The *Bgl*II-*Bam*HI-generated fragments from plasmid pJD2107 were inserted into the unique *Bgl*II restriction site of plasmid pDM306 to generate plasmids pJD2771 and pJD2772. For sequencing analysis, the 3.7-kb *Bgl*II-7 fragment of plasmid pJD2107 was inserted into the *Bam*HI restriction site of pUC-derivative pSVB30 (Arnold and Pühler 1988), yielding pSVB:B7α and pSVB:B7β. The 0.7-kb *Hind*III-*Eco*RI fragment from pSVB:B7β was inserted into *E. coli*-*Clavibacter* shuttle vector pDM302, resulting in

plasmid pJD1672. Plasmid pJD1572 was constructed by inserting a 1.5-kb *Hind*III-*Sma*I restriction fragment from pSVB:B7β containing the *pat*-1 region into *Hind*III-*Sca*I-digested pDM302. The 1.1-kb *Bam*HI-*Sma*I restriction fragment from pSVB:B7β, treated with Klenow polymerase, was purified from agarose gels by the Gene-clean-II kit (Dianova, Hamburg, Germany), and ligated into the *Sca*I restriction site of plasmid pDM302 to generate plasmid pJD2773. Additional deletions of the *pat*-1 region were obtained by subcloning

Fig. 7. Secondary structure of 3' end of *pat*-1 transcript. Repetitive sequence *pat*-1_{rep} (nucleotide position 1363 to 1569) can form a stable fold-back structure, consisting of a stem-loop and a terminal rho-independent terminator.

ExoIII/S1 deletion fragments into vector pDM302 to form plasmids pJD1671 and pJD1676.

Polymerase chain reaction.

Polymerase chain reactions were carried out with the GeneAmp DNA Amplification Kit and a DNA thermal cycler, both from Perkin-Elmer Cetus (Norwalk, CT). In general, approximately 50 ng of template DNA and 20 pmol of each primer DNA were used per reaction. The set of synthetic oligonucleotide primers used had the following nucleotide sequence: 5'-GGG AGT ACT GAA TTC GTA CCC CGA GAA CCG GGG-3' (P1rep; nucleotide sequence position 1358) and 5'-GGG AGT ACT AAG CTT GCG CCC GTG TCG AAC ATT- 3' (P3rep; nucleotide sequence position 1577). They were deduced from nucleotide sequence information of the *pat-1* region, and generated a 219-bp PCR amplification product. Generally, 25 PCR cycles were run with denaturation at 94°C for 1 min, annealing at 53°C for 2 min, and extension at 72°C for 2 min.

DNA sequencing.

The DNA sequence of both strands was determined by the dideoxy chain termination method (Sanger et al. 1977), with the DNA sequencing kit, TAQuence, according to the manufacturer's protocol (United States Biochemical, Cleveland, OH). Sequencing reactions were carried out on alkali denatured plasmid DNA as template with *Taq* DNA polymerase and Deaza Nucleotide Mixtures. For nucleotide sequence determination the uni-directed deletions of plasmids pSVB:B7 α and pSVB:B7 β were generated after thionucleotide end protection followed by successive action of exonuclease III of *E. coli* and nuclease S1 of *Aspergillus oryzae* with the double-stranded nested deletion Kit (Pharmacia LKB Biotechnology, Freiburg, Germany). DNA sequence analyses were performed with the computer software packages Microgenie (Beckman Instruments, Fullerton, CA) and PCgene (Intelligenetics, Geneva), respectively. Protein sequences deduced from the nucleotide sequence were compared with the NBRF- and Swiss-Protein sequence databases.

Clavibacter RNA preparation.

Total RNA was isolated from *C. michiganensis* subsp. *michiganensis* strains essentially as described by Sambrook et al. (1989) with the following modifications: Cells were grown in 80 ml of LB medium up to a titer of approximately 10^8 cells per ml and collected by centrifugation at $3,000 \times g$ for 10 min at 4°C. Bacteria were suspended in 3.5 ml of ice-cold 4 M guanidinium isothiocyanate buffer containing 0.05% Sarkosyl NL-30 (Serva, Heidelberg, Germany), 1 % β -mercaptoethanol, and 0.1 M Tris-HCl, pH 7.5. The cell homogenate was shock frozen in liquid nitrogen, thawed, and lysed by French press treatment at 18,000 lb/in² until a homogeneous solution resulted. Solutions used for RNA purification were treated with 0.1% diethylpyrocarbonate (DEPC; Sigma Chemical, St. Louis, MO) as described (Sambrook et al. 1989). Cell homogenate (3 ml) was layered onto a 1.5-ml 5.7 M CsCl solution containing 100 mM EDTA (pH 7.0) in a polyallomer tube (Beckman Instruments) and then centrifuged at 35,000 rpm (rotor Tst55.5; Beckman) for 18 h at 20°C. The pelleted RNA was resuspended in 200 μ l of ice-cold, distilled H₂O (0.1% DEPC). The RNA suspension was precipitated

with 2 volumes of ethanol and 1/10 volume 1 M LiCl at -70°C. After two washing steps with 80% ethanol the RNA was dissolved in DEPC-treated H₂O (0.1% DEPC). An average yield of about 2 mg of RNA was achieved from 80-ml cultures of *Clavibacter*.

Northern transfer and hybridization.

Denatured RNAs were separated by formaldehyde agarose gel electrophoresis as described by Sambrook et al. (1989). The 0.24- to 9.5-kb RNA ladder obtained from Gibco/BRL (Berlin) was used as the molecular weight standard. RNAs were transferred to Hybond N nylon membranes (Amersham, Braunschweig, Germany) with 10 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 15 mM sodium citrate), using a LKB 2016 VacuGene blotting apparatus. Prehybridizations were performed at 42°C for 6 h in a solution of 5 $\times$ SSC, 50% formamide, 50 mM phosphate buffer, pH 6.7, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mg of denatured calf thymus DNA per ml; DNA probes were labeled with [α -³²P]dATP by random priming with the random-primed DNA labeling kit supplied by Boehringer Mannheim (No. 1004 760). An internal 0.6-kb PCR product of the ORF1 gene, generated with primers CMM-5 and CMM-6 (*pat-1* nucleotide position 411 to 1019) was used as a probe (Dreier et al. 1995). The denatured probe was added to the prehybridization solution, followed by gentle agitation at 42°C for 16 h. The nylon membranes were washed twice for 10 min at room temperature in a 2 $\times$ SSC, 0.1% SDS solution, and twice for 15 min at 68°C in a solution consisting of 0.1 $\times$ SSC and 0.1% SDS. The hybridization results were visualized by autoradiography.

Primer extension.

The mapping of the 5' end of the mRNA was done by primer extension analysis. The primer used in cDNA synthesis and DNA sequencing was a synthetic 35-mer oligonucleotide (5'-GCG ACA TGA ACT GCA TAG CGC CCG GAA CAG GC-3') complementary to the 5' end of the ORF1. Total cellular RNA (15 μ g) was hybridized with the DNA primer (2 ng). Annealing reactions were carried out in DEPC-treated H₂O by boiling the reactions for 5 min, followed by slow cooling to 42°C. First-strand cDNA synthesis was accomplished by incubating the primer-template complex at 42°C for 15 min with 100 U of Superscript (Gibco/BRL, Berlin), 20 U RNasin (Promega, Madison, WI), 4 μ l of 5 $\times$ reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl₂), 2 μ l of 100 mM dithiothreitol, 2 μ l of mixed dNTP stock (2.5 mM each dGTP, dCTP, dTTP), and 1 μ l of [α -³²P]dATP (0.4 MBq). Reactions were chased with 2 μ l of 2.5 mM dATP at 37°C for 15 min and terminated by the addition of an equal volume of a mixture containing 95% formamide, 10 mM NaOH, 0.05% bromophenol blue and cyanol blue. For DNA sequencing, the same oligonucleotide primer was used (16 ng per reaction). Samples of the sequencing and cDNA reaction products were boiled for 3 min, loaded on a 6% polyacrylamide/7 M urea gel (Garoff and Ansorge 1981), and separated by electrophoresis to determine the size of the primer extension products.

Virulence assay.

The standard infection procedure was carried out as described previously (van Steekelenburg 1985). Four-week-old

tomato plants (*L. esculentum* cv. Moneymaker) were infected by cutting off the petiole of the first true leaf near the stem with a scalpel that had been dipped into the bacterial suspension to be tested. The virulent phenotype was estimated by determination of leaf wilting symptoms and plant biomass. Plant biomass corresponding to the dry weight of 30 plants was determined 4 weeks after infection.

In order to test for the possibility that virulence is correlated with the ability of the bacteria to colonize the host plant, the titer of viable bacteria in the plant tissue was determined at different times after infection. Tomato plants were homogenized by grinding in a mortar after addition of 10 ml of K-phosphate buffer, pH 7.2, serial dilutions were plated on modified SCM medium semiselective for *Clavibacter*, and colonies were scored after incubation at 24 to 26°C for 5 days.

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