Identification of a Tomatinase in the Tomato-Pathogenic Actinomycete *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382

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The insertion site of a transposon mutant of *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 was cloned and found to be located in the gene *tomA* encoding a member of the glycosyl hydrolase family 10. The intact gene was obtained from a cosmid library of *C. michiganensis* subsp. *michiganensis*. The deduced protein TomA (543 amino acids, 58 kDa) contains a predicted signal peptide and two domains, the N-terminal catalytic domain and a C-terminal fibronectin III-like domain. The closest well-characterized relatives of TomA were tomatinases from fungi involved in the detoxification of the tomato saponin α -tomatine which acts as a growth inhibitor. Growth inhibition of *C. michiganensis* subsp. *michiganensis* by α -tomatine was stronger in the *tomA* mutants than in the wild type. Tomatinase activity assayed by deglycosylation of α -tomatine to tomatidine was demonstrated in concentrated culture supernatants of *C. michiganensis* subsp. *michiganensis*. No activity was found with the *tomA* mutants. However, neither the transposon mutant nor a second mutant constructed by gene disruption was affected in virulence on the tomato cv. Moneymaker.

Clavibacter michiganensis subsp. *michiganensis* causes the bacterial wilt of tomato. After infection, *C. michiganensis* subsp. *michiganensis* NCPPB382 spreads systemically in the xylem of the plants. Various infection modes are described (Strider 1969): contaminated seed; natural or artificial wounds of roots, stems, or leaf trichomes; and infection through stomata. Infection through hydathodes also was observed (Carlton et al. 1998). The progressive disease leads to wilting and tissue macerations causing lesions of the infected stems (bacterial canker).

One of the plant defenses to which *C. michiganensis* subsp. *michiganensis* is exposed in the infected tomato plant is the production of saponines, consisting mainly of α -tomatine and dehydrotomatine (Kozukue et al. 2004). α -Tomatine is produced in all parts of the plant, but the content varies among different plant tissues, with the highest concentration in green fruit and leaves and the lowest in roots (Friedman and Levin 1998; Kozukue et al. 2004). The α -tomatine content is further enhanced after recognition of pathogens by the plant (Arwiyanto

et al. 1994; Smith and MacHardy 1982). α -Tomatine inhibits fungal growth (Roldan-Arjona et al. 1999; Sandroock and VanEtten 1998), and inhibition of bacterial growth also has been observed (Arwiyanto et al. 1994; El-Shanshoury et al. 1995). The alkaloid α -tomatine is composed of the steroidal aglycone tomatidine and the tetrasaccharide β -lycotetraose consisting of two glucose, one galactose, and one D-xylose residues (Fig. 1). For several species of fungi, detoxification mechanisms were described which involve the production of tomatinases of differing specificities that remove sugar moieties from α -tomatine (Fig. 1) (Osborn 1996; Quidde et al. 1998; Roldan-Arjona et al. 1999). In this study, we describe the identification of a bacterial tomatinase in the tomato pathogen *C. michiganensis* subsp. *michiganensis*.

RESULTS

Characterization of the transposon insertion site in a gene encoding a tomatinase.

After transposon mutagenesis of *C. michiganensis* subsp. *michiganensis* NCPPB382 using the artificial transposon Tn1409C β , which carries a chloramphenicol resistance gene *cmx* (Gartemann and Eichenlaub 2001), the insertion site of one mutant was characterized. From one of the first mutants obtained, the restriction fragments of *C. michiganensis* subsp. *michiganensis* C7 were cloned and sequence analysis of the resulting plasmids pKGT1040 and pTT1, containing the insertion site of Tn1409C β (Fig. 2), revealed that the inactivated gene, designated *tomA*, encoded a member of family 10 of glycosyl hydrolases (Henrissat 1991). Most members of this family are endoxylanases. However, the best similarity obtained was to α -tomatinases of *Fusarium oxysporum* f. sp. *lycopersici* strains, also a vascular pathogen of tomato (Fig. 3). This fungal enzyme catalyses the removal of the complete tetrasaccharide lykotetraose of α -tomatine (Roldan-Arjona et al. 1999).

In our cosmid library of *C. michiganensis* subsp. *michiganensis*, we identified *tomA* in the cosmid clone C2-28 by colony hybridization against a labeled *tomA* probe. Subclones of C2-28 termed pIG1 and pIG2 were constructed in pUC18 after hydrolysis with *EcoRI* and *PstI* (Fig. 2). The complete nucleotide sequence of the 6.1-kb fragment represented by the inserts of these two plasmids was determined. Five open reading frames were identified. Only the 5' part of the *regA* gene was present, followed by *regB* encoding a sensor kinase and a response regulator of a two-component regulatory system. The genes are located directly upstream of *tomA*. Downstream of *tomA*, the *catR* gene codes for a regulatory protein of 342 amino

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acids (36 kDa), which is a member of the LacI-repressor family. Another gene, *bglA*, encodes a putative β -glucosidase (481 amino acids, 53 kDa) of unknown specificity.

The *tomA* gene codes for a putative tomatinase, a protein of 543 amino acids (58 kDa). At the N-terminus, there is a leader peptide (amino acids 1 to 27), indicating secretion of the protein. TomA has two domains, a catalytic domain (amino acids 28 to 340) and a second domain (amino acids 340 to 543), which exhibit weak similarity to PKD- or fibronectin III-like domains. The catalytic domain displays an identity or similarity of approximately 55 and 70% of the amino acids residues

to the tomatinases of several *Fusarium oxysporum* strains and a protein from *Aspergillus nidulans*, respectively (Fig. 3). On the other hand, the identity or similarity values to the most closely related xylanases originating from bacteria are much lower, at approximately 35 and 50%, respectively (Fig. 3), suggesting that *tomA* might encode a bacterial tomatinase.

The C-terminal fibronectin III-like domain is related to similar domains occurring in glycosyl hydrolases of bacteria (Howard et al. 2003; Saito et al. 2003; Zverlov et al. 1998). The exact function of the domain in these proteins is unknown. This putative fibronectin III-like domain is missing in both the

Fig. 1. Structure of α -tomatine. The cleavage sites of the tomatinases from *Fusarium oxysporum* f. sp. *lycopersici* (Fol) (Roldan-Arjona et al. 1999), *Septoria lycopersici* (Sl) (Martin-Hernandez et al. 2000), and *Botrytis cinerea* (Bc) (Quidde et al. 1998) are indicated.

Fig. 2. Physical map of the chromosomal region carrying the *tomA* gene of *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382. Genes and directions of transcription are represented by arrows. Bars indicate regions cloned into plasmids used in this study. The insertion site of Tn1409 in the transposon mutant *C. michiganensis* subsp. *michiganensis* C7 is indicated. The second *SphI*, respectively, *BglII* site of pKGT1040 and pTT1, lies inside of Tn1409C and is not shown. The restriction sites given for pKGC33 and pOKA1 α are the sites used to insert the *cmx* cassette.

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tomatinase and the xylanases of *F. oxysporum*. Only the recently described tomatinase homolog from *Streptomyces turgidiscabies* (Kers et al. 2005) contains both domains in the same order as in *C. michiganensis* subsp. *michiganensis*, with high sequence similarity and identity of 58 and 71% of the amino acid residues, respectively, over the whole protein.

Distribution of *tomA* in various *C. michiganensis* strains.

Total DNAs of several *C. michiganensis* strains were probed for the presence of *tomA* using a 1.6-kb *SphI/NheI* digoxigenin-11-UTP-labeled probe derived from pIG2 (Fig. 2). With CMM100, a plasmid-free derivative of *C. michiganensis* subsp. *michiganensis* NCPPB382 (Meletzus and Eichenlaub 1991), a hybridization signal was obtained, demonstrating that *tomA* maps on the chromosome. All other *C. michiganensis* subsp. *michiganensis* strains tested also carry a *tomA* homolog, with the exception of NCPPB3264. No signal was obtained for the other *Clavibacter* subspecies, *C. michiganensis* subsp. *sepedonicus* (*Solanum tuberosum*), *nebraskensis* (*Zea mais*), *tesselarius* (*Triticum aestivum*), and *insidiosus* (*Medicago sativa*) (Fig. 4). Strains of the related plant pathogens *Curtobacterium flaccumfaciens* pv. *oortii*, three *Rathayibacter* spp., and *Arthrobacter ilicis* gave no positive signal with the *tomA* probe (data not shown).

Insertional inactivation of *tomA*.

In the course of the development of genetic procedures for *Clavibacter michiganensis* subsp. *michiganensis*, a second *tomA* mutant was constructed via gene replacement by substitution of a 1.5-kb internal fragment of *tomA* with a chloramphenicol resistance gene. The 3.3-kb *EcoRI/BglII* fragment of pIG2 carrying *tomA* was cloned into pUC13 (pKGC33) and the *BamHI* fragment internal of *tomA* was replaced by the 1.5-kb *BamHI* fragment of pOKUCmB α carrying the *cmx* gene (Fig. 2). The resulting hybrid plasmid pOKA1 α carries both a large deletion inside of *tomA* and the selectable marker gene *cmx* in the same orientation as *tomA*. Chloramphenicol-resistant transformants were selected and replacement of the chromosomal *tomA* by the inactivated gene copy via homologous recombination was confirmed by Southern blot analysis (Fig. 5). The 3.9-kb *EcoRI/PstI* fragment from pIG2 was used as a *tomA*-specific hybridization probe. *PstI*-digested total DNA of *C. michiganensis* subsp. *michiganensis* NCPPB382 showed a single hybridizing restriction fragment of 6.1 kb when probed with the *tomA*-specific hybridization probe. Transformants in which the chromosomal *tomA* was replaced by double cross-

over events have an additional *PstI* restriction site inside the *cmx* gene leading to two hybridizing signals of 2.9 and 3.2 kb. To exclude the possibility that the whole suicide plasmid had integrated into the chromosome of *C. michiganensis* subsp. *michiganensis*, a hybridization against a pUC13 probe was conducted (data not shown). Thus, three *tomA* mutants (*C. michiganensis* subsp. *michiganensis* Tom2, Tom3, and Tom10) out of 20 chloramphenicol-resistant clones examined were obtained (Fig. 5). *PstI*-digested total DNA of the transposon mutant *C. michiganensis* subsp. *michiganensis* C7 gave two hybridizing restriction fragments of 2.3 and 4.4 kb when probed with the *tomA*-specific hybridization probe. The 3.4-kb transposon inserted in *tomA* carries five additional *PstI* restriction sites but only the *PstI* fragments carrying the ends of the transposon (0.5 kb from the 5' and 1.3 kb from the 3' end of *tomA*) hybridize with the probe. This leads to fragments of 2.3 and 4.4 kb in length that show signals by Southern hybridization (Fig. 5).

Plasmid pCM2 of strain NCPPB382 is unstable; therefore, the *tomA* mutants (Tom2, Tom3, Tom10, and C7) were tested for their plasmid status. By Southern hybridization, it was

Fig. 5. Southern blot analysis of *Clavibacter michiganensis* subsp. *michiganensis* *tomA* mutants. *PstI*-digested total DNA of *C. michiganensis* subsp. *michiganensis* strains was hybridized against a 3.9-kb *EcoRI/PstI* *tomA* probe. Lane 1, digoxigenin-labeled λ *EcoRI/HindIII*; lanes 2 to 4, gene replacement mutants *C. michiganensis* subsp. *michiganensis* Tom2, Tom3, and Tom10, respectively; lane 5, transposon mutant C7; and lane 6, NCPPB382 (wild type).

Fig. 4. Southern blot of total DNA of various *Clavibacter michiganensis* strains digested with *KpnI* and hybridized against an internal 1.6-kb *SphI/NheI*-fragment of *tomA*. *C. michiganensis* subsp. *michiganensis* strains used were as follows. Lane 1: *tesselarius* ATCC33566; lane 2, *insidiosus* NCPPB1109; lane 3, *nebraskensis* NCPPB2526; lane 4: *sepedonicus* CS7; lane 5, CMM100; lane 6, *michiganensis* NCPPB382; lane 7, *michiganensis* C7; lane 8, *michiganensis* NCPPB3264; lane 9, *michiganensis* NCPPB2941; lane 10, *michiganensis* NCPPB2979; lane 11, *michiganensis* NCPPB3342; lane 12, *michiganensis* NCPPB3123; lane 13, *michiganensis* NCPPB1574; lane 14, *michiganensis* NCPPB2943; lane 15, *michiganensis* LMG5602; and lane 16, digoxigenin-labeled λ *EcoRI/HindIII*.

shown that all the mutants carried only plasmid pCM1 with the pathogenicity gene *celA* (Jahr et al. 2000) (data not shown). Thus, strain CMM101, which also carries only pCM1, was used as a control in plant assays which were performed to determine whether TomA is affecting virulence. Infection of tomato plants with the *tomA* gene replacement mutants (Tom2, Tom3, and Tom10) and the transposon mutant (C7) did not show any significant differences in the extent of virulence compared with CMM101. The wilting index determined by three independent tests was 14.1 ± 1.0 days for the *tomA* mutants and did not differ significantly from the control strain CMM101 with 14.3 ± 0.9 days. Also, the bacterial titer in planta of the *tomA* mutants ($7.24 \pm 4.30 \times 10^9$ CFU/g of plant homogenate) 28 days after infection was in the same range as for the wild-type control, CMM101 ($5.70 \pm 2.14 \times 10^9$ CFU/g of plant homogenate). This indicates that, in the interaction with the tomato cv. Moneymaker, *tomA* is not a virulence factor.

Determination of tomatinase activity.

Because we were unable to demonstrate an effect of *tomA* on virulence, it was necessary to show that *tomA* really encodes a tomatinase. The tomatinase-deficient mutants *C. michigan-*

ensis subsp. *michiganensis* Tom2 (gene replacement mutant) and C7 (transposon mutant) and the wild-type strain CMM101 were grown in TBY (tryptone at 10 g/liter, yeast extract at 5 g/liter, NaCl at 5 g/liter, pH 7.5) containing 0.02 mM α -tomatine. Both culture supernatants and crude protein extracts of these strains were tested for their ability to deglycosylate α -to-

Table 1. Inhibition of growth of *Clavibacter michiganensis* subsp. *michiganensis* by α -tomatine^a

α -Tomatine (μ mol)	Inhibition zone diameter (mm)		Inhibition (%) ^c
	CMM101 ^b	Tom2 ^b	
0 ^d	...	...	...
0.05	6.0 ± 0	6.8 ± 0.4	12.5
0.10	7.6 ± 0.7	11.0 ± 0.4	45.1
0.20	11.6 ± 0.5	17.3 ± 0.4	49.0
0.30	15.1 ± 0.6	22.7 ± 1.0	50.3

^a Means and standard deviations are from five independent assays.

^b CMM101, pCM1; Tom2, pCM1, *tomA* gene replacement mutant.

^c Increase of inhibition. Given is the percentage by which the growth inhibition in the mutant is higher.

^d Control: Na-citrate buffer.

Fig. 6. Thin-layer chromatography analysis of tomatinase reaction products. Supernatants and crude protein extracts from *Clavibacter michiganensis* subsp. *michiganensis* strains were tested for α -tomatine degradation. Lane 1 to 3, crude extracts from CMM101 (wild type), *C. michiganensis* subsp. *michiganensis* Tom2, and C7, respectively; lanes 4 to 6, concentrated supernatants from CMM101 (wild type), Tom2, and C7, respectively; lane 7, mixture of 0.1 mM α -tomatine and tomatidine.

Fig. 7. Growth inhibition of α -tomatine in an agar plate diffusion assay. Paper disks contain Na-citrate buffer (control = 1), or increasing amounts of α -tomatine (2 = 0.05 μ mol, 3 = 0.1 μ mol, 4 = 0.2 μ mol, 5 = 0.3 μ mol) dissolved in Na-citrate buffer.

matine by thin-layer chromatography (TLC) analysis (Fig. 6). In the supernatant of strain CMM101 carrying the wild-type *tomA* gene, we found tomatinase activity with conversion of α -tomatine to tomatidine, indicating that the enzyme was secreted and that tomatinase removes the complete lycotetraose from α -tomatine. Thus, TomA seems to have the same activity as the homologous tomatinase from *F. oxysporum*, which carries out the same reaction producing tomatidine (Roldan-Arjona et al. 1999). Supernatants of both *tomA* mutants did not show any tomatinase activity. *C. michiganensis* subsp. *michiganensis* NCPPB382 showed the same degrading α -tomatine activity in TLC experiments as CMM101 (data not shown).

Interestingly, crude protein extracts from mutants and CMM101 had a deglycosylation activity converting α -tomatine to tomatidine. This degradation probably is caused by unspecific glycosidases present in the cytoplasm of *C. michiganensis* subsp. *michiganensis*.

Inhibition of bacterial growth by α -tomatine.

Finally, we tested whether α -tomatine has an inhibitory effect on growth of *C. michiganensis* subsp. *michiganensis*. In diffusion tests on agar plates with α -tomatine (0.05 to 0.3 μ mol), both mutants, Tom2 and C7, were found to be more sensitive to α -tomatine than the control strain CMM101. Both wild-type and mutants showed almost no inhibition of growth in the buffer control and with 0.05 μ mol α -tomatine. For higher concentrations of α -tomatine, a concentration-dependant increase in growth inhibition was observed for all strains. Compared with the control strain CMM101, the mutants were affected

much more strongly (Table 1; Fig. 7). For α -tomatine at 0.1 μ mol, the growth inhibition zone of the gene replacement mutant was approximately 45% larger and, at 0.3 μ mol, the highest concentration of α -tomatine tested, the inhibition zone was approximately 50% larger than the corresponding ones of strain CMM101 (Fig. 7). This indicates that growth inhibition of *C. michiganensis* subsp. *michiganensis* caused by α -tomatine occurs and that TomA can detoxify α -tomatine. As in noninfected tomato plants of cv. Moneymaker, the content of α -tomatine is approximately 775 nmol/g of fresh weight (Beimen et al. 1992), the amounts of α -tomatine used in the inhibition test (50 to 300 nmol) cover the physiological relevant range.

To determine the inhibitory concentration of α -tomatine more precisely, *C. michiganensis* subsp. *michiganensis* was grown in liquid TBY medium with different α -tomatine concentrations. However, because both wild-type and *tomA* mutants formed large aggregates when α -tomatine was present in concentrations above 0.2 mM, it was impossible to determine exact bacterial titers. Bacterial aggregation may be due to the production of polysaccharides, which has to be investigated in future work.

DISCUSSION

Here we describe the identification of a bacterial tomatinase involved in the detoxification of α -tomatine, a phytoanticipin produced by tomato as a defense against pathogen infection.

The toxicity of α -tomatine against *C. michiganensis* subsp. *michiganensis* could be demonstrated, which agrees with

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source ^b
Strains		
<i>Clavibacter michiganensis</i>		
subsp. <i>michiganensis</i> NCPPB382	pCM1, pCM2; virulent on tomato	NCPPB
subsp. <i>michiganensis</i> CMM101	Curing derivative of NCPPB382, pCM1, virulent on tomato	Meletzus and Eichenlaub 1991
subsp. <i>michiganensis</i> CMM100	Curing derivative of NCPPB382, plasmid-free; avirulent on tomato	Meletzus and Eichenlaub 1991
subsp. <i>michiganensis</i> CmmC7	pCM1; <i>tomA</i> containing a Tn1409C insertion; Cm ^r	This work
subsp. <i>michiganensis</i> CmmTom2	pCM1; <i>tomA</i> gene replacement mutant; Cm ^r	This work
subsp. <i>michiganensis</i> NCPPB3264	Highly virulent on tomato	NCPPB
subsp. <i>michiganensis</i> NCPPB2979	Virulent on tomato	NCPPB
subsp. <i>michiganensis</i> NCPPB3342	Virulent on tomato	NCPPB
subsp. <i>michiganensis</i> NCPPB3123	Avirulent on tomato	NCPPB
subsp. <i>michiganensis</i> NCPPB1574	Avirulent on tomato	NCPPB
subsp. <i>michiganensis</i> NCPPB2941	Virulent on <i>Capsicum frutescens</i>	NCPPB
subsp. <i>michiganensis</i> NCPPB2943	Virulent on <i>Capsicum frutescens</i>	NCPPB
subsp. <i>michiganensis</i> LMG5602	Virulent on <i>Cyphomandra betacea</i>	LMG
subsp. <i>sepedonicus</i> CS7	Virulent on potato (<i>Solanum tuberosum</i>)	Laine et al. 1996
subsp. <i>nebraskensis</i> NCPPB2526	Virulent on corn (<i>Zea mays</i>)	NCPPB
subsp. <i>insidiosus</i> NCPPB1109	Virulent on alfalfa (<i>Medicago sativa</i>)	NCPPB
subsp. <i>tessellari</i> ATCC33566	Virulent on wheat (<i>Triticum aestivum</i>)	ATCC
<i>Escherichia coli</i> JM109	<i>recA1 endA1 thi hsdR17 supE44 relA1 Δ(lac-proAB) gyrA96 F'(traD36 proAB⁺ lacP⁺lacZΔM15)</i>	Yanisch-Perron et al. 1985
<i>E. coli</i> GM119	F ⁻ <i>dcm-6 dam-3 metβ1 galK2 galT22 lacY1 tsx-78 supE44</i>	W. Wackernagel
<i>E. coli</i> NM554	F ⁻ <i>recA araD Δ(ara,leu) ΔlacY galU galK hsr⁺ hsm⁺ strA mcrA mcrB</i>	Stratagene, La Jolla, CA, U.S.A.
Plasmids		
pUC13	Cloning vector; Ap ^r	Vieira and Messing 1982
pUC18	Cloning vector; Ap ^r	Yanisch-Perron et al. 1985
pKGT452Cβ	Tn1409Cβ; <i>mpA</i> and <i>cmx</i> in the same orientation	Gartemann and Eichenlaub 2001
pKGT1040	pUC18::3.8-kb <i>SphI</i> fragment of mutant C7	This work
pTT1	pUC13::5.5-kb <i>BglII</i> fragment of mutant C7	This work
SuperCos1	Cosmid; Ap ^r , Km ^r	Stratagene
C2-28	SuperCos1::35-kb fragment of NCPPB382; <i>tomA</i>	This work
pIG1	pUC18::2.2-kb <i>EcoRI/PstI</i> fragment of C2-28	This work
pIG2	pUC18::3.9-kb <i>EcoRI/PstI</i> fragment of C2-28; <i>tomA</i>	This work
pOKU9cmBα	pUC18::1.5-kb <i>BamHI</i> fragment carrying <i>cmx</i>	This work
pKGC33	pUC13::3.3-kb <i>BglII/EcoRI</i> fragment of pIG2, <i>tomA</i> ; Ap ^r	This work
pOKA1α	pKGC33, <i>cmx</i> replacing the internal 1.5-kb <i>BamHI</i> -fragment of <i>tomA</i> ; Ap ^r , Cm ^r	This work

^a Ap^r = ampicillin resistance, Cm^r = chloramphenicol resistance, and Km^r = kanamycin resistance.

^b NCPPB = National Collection of Plant Pathogenic Bacteria, U.K.; LMG = Laboratorium voor Microbiologie, Gent, Belgium; ATCC = American Type Culture Collection, Manassas, VA, U.S.A.; W. Wackernagel, University of Oldenburg, Oldenburg, Germany.

earlier reports on growth inhibition of bacteria by α -tomatine (Arwiyanto et al. 1994; El-Shanshoury et al. 1995). The basis for this toxicity is unknown. For fungi, an interaction of α -tomatine with membrane steroids leading to disruption of membranes is discussed (Osborn 1996). Bacteria do not possess membrane steroids; therefore, a reason for toxicity against bacteria remains to be explained. The amphipatic nature of α -tomatine, with its hydrophilic tetrasaccharide component and hydrophobic steroid-like aglycone, may allow insertion into the membrane. Because the *C. michiganensis* subsp. *michiganensis* tomatinase mutants were more sensitive to α -tomatine than the control strain CMM101, a role of TomA in detoxification seems possible.

The tomatinase may facilitate invasion of plant tissues by *C. michiganensis* subsp. *michiganensis*; however, we find that the *tomA* mutants can infect and grow normally in the tomato cv. Moneymaker and are not reduced in virulence. Similar conflicting phenotypes were reported for fungi where a detoxification of α -tomatine also occurs, but mutants are not or only slightly affected in virulence (Martin-Hernandez et al. 2000; Quidde et al. 1998). It is possible that, although the tomatinase is not important in cv. Moneymaker, it may be so in tomato cultivars which produce a larger amount of α -tomatine. This seems to be the case especially in the related wild tomato species (e.g., *Lycopersicon peruvianum* and *L. hirsutum*) (Masahiro et al. 1996), which could represent a natural reservoir for virulent *C. michiganensis* subsp. *michiganensis* strains. The tomato cultivars developed for agricultural food production mostly have a low α -tomatine content compared with wild tomato species (Masahiro et al. 1996). The amount of α -tomatine present in Moneymaker (Beimen et al. 1992) is in the same range as reported for other cultivated tomato lines, which display a high variability in concentration among both races and different tissues (Friedman and Levin 1998; Kozukue et al. 2004; Osborn 1996).

On the other hand, it may be possible that our infection method, which provides *C. michiganensis* subsp. *michiganensis* a direct access to the xylem where the α -tomatine content is low, as long as no induction of α -tomatine production due to recognition of the pathogen occurs (Smith and MacHardy 1982), circumvents this defense mechanism. Further studies, such as the comparison of virulence using tomato cultivars with higher α -tomatine content and less drastic infection procedures, are required to give an answer to this question.

Interestingly, an entirely different function in pathogenic interaction may be ascribed to the tomatinase. It recently was reported by several groups that degradation products of α -tomatine suppress induced plant defense mechanisms such as hypersensitive response (Bouarab et al. 2002; Ito et al. 2004). Thus, it is possible that the tomatinase is one of several factors which prevent plant defense in the *C. michiganensis* subsp. *michiganensis*–tomato interaction.

The catalytic domain of TomA belongs to family 10 of glycosyl hydrolases. We were able to demonstrate tomatinase activity for TomA. When comparing the sequences, a clear distinction between enzymes of proven endo-xylanase activity and enzymes closely related to TomA is seen. However, because some of these close relatives originate from organisms which are non-pathogenic (e.g., *Aspergillus* spp.) or have hosts which do not produce α -tomatine (e.g., *S. turgidiscabies*), the substrate specificity of the enzyme remains interesting. Also, we cannot rule out that TomA has a cryptic xylanase activity. Therefore, we currently are trying to express TomA in *Escherichia coli* and to purify the protein, which will enable the determination of substrate specificity toward related alkaloids and xylan.

So far, we have no evidence that the neighboring *regAB* and *catR* genes are involved in the regulation of *tomA*. However, we

are constructing mutants for these genes and will investigate a possible role for them in the future. Also, there is no indication that the putative β -glucosidase encoded by *bglA* is involved in α -tomatine degradation because no leader peptide was predicted and the protein seems to be located in the cytoplasm.

MATERIALS AND METHODS

Media and growth conditions.

Bacterial strains and plasmids used and constructed in this study are listed in Table 2. *E. coli* was grown on TBY at 37°C. *Clavibacter* strains were grown at 26°C on TBY. SB medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, NaCl at 5 g/liter, 0.5 M sorbitol, 20 mM CaCl₂, 20 mM MgCl₂) was used for regeneration of *C. michiganensis* subsp. *michiganensis* after electroporation. For recombinant *E. coli* strains carrying pUC derivatives, ampicillin (150 µg/ml) or chloramphenicol (10 µg/ml) was added. *Clavibacter* strains bearing the *cmx* gene were grown in the presence of chloramphenicol (10 µg/ml).

DNA isolation, DNA manipulation, and DNA transfer.

Plasmid DNA of *E. coli* was prepared by the alkaline lysis method (Sambrook et al. 1989). Plasmid DNA used for sequencing was isolated and purified by QIAGEN columns as specified by the manufacturer (Qiagen, Hilden, Germany). Preparation of total DNA for hybridization experiments and the construction of the cosmid library was done according to Hopwood and colleagues (1985). DNA restriction, fill-in reactions, ligation, and transformation were carried out following standard procedures (Sambrook et al. 1989). Isolation of restriction fragments from agarose gels was done with the JETSORB kit as specified by the manufacturer (Genomed, Bad Oeynhausen, Germany). Electroporation of *C. michiganensis* subsp. *michiganensis* was conducted as described by Kirchner and associates (2001) with the modification that only 1.25% glycine was used in the pretreatment of the cells.

Insertional inactivation of the *tomA* gene.

pKGC33 contains only two *Bam*HI restriction sites inside of *tomA*. After hydrolysis with *Bam*HI, the 1.5-kb internal *tomA* fragment was removed and replaced by a 1.5-kb *Bam*HI fragment from pOKU9cmB α which contains a *cmx* resistance gene. This resulted in the gene replacement vector pOKA1 α which contains the *cmx* resistance gene in the same orientation as *tomA*.

Southern blot analysis.

Probes for hybridization were isolated from agarose gels and labeled with digoxigenin-11-dUTP using the Random Primed DNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). As a probe for the *cmx* gene (Tauch et al. 1998) specifying the chloramphenicol exporter, a 2.2-kb *Sph*I/*Bgl*II-fragment of pKGT452C β was used. The *tomA* gene was probed with the 1.6-kb *Sph*I/*Nhe*I fragment of pIG2. Also, the 3.9-kb *Eco*RI/*Pst*I fragment from pIG2 was used as a *tomA*-specific hybridization probe for identification of *tomA* gene replacement mutants. Digested chromosomal and plasmid DNA samples were separated on 0.7 to 1.0% agarose gels and transferred to a nylon membrane (porablot; Macherey & Nagel, Düren, Germany) by capillary blots (Smith and Summers 1980). Hybridizations were carried out at 68°C overnight in a buffer containing 5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% sodium dodecyl sulfate (SDS), 0.1% Na-laurylsarcosyl, and 2% blocking reagent (Roche Diagnostics). The nylon membrane was washed twice in 0.1 $\times$ SSC and 0.1% SDS at 68°C for 15 min. Detection was carried out as recommended by the manufacturer (Roche Diagnostics).

DNA sequencing.

For DNA sequencing, the dideoxy chain termination method (Sanger et al. 1977) was used. Sequencing was done by the IIT-Biotech GmbH (Bielefeld, Germany) using an ABI prism 377 apparatus (Applied Biosystems, Foster City, CA, U.S.A.). For sequencing, the plasmids pKGT1040, pTT1, pIG1, and pIG2 were used. The complete nucleotide sequence was determined by primer walking using synthetic oligonucleotide primers obtained from Sigma Genosys (Darmstadt, Germany). The overlap between pIG1 and pIG2 was obtained by sequences from pTT1. The DNA and deduced proteins sequences were compared with database entries using the BLAST programs (Altschul et al. 1997) at the server of the National Center of Biotechnology Information. Multiple sequence alignments were performed with ClustalX 1.83 (Thompson et al. 1994).

Preparation

of crude extracts and cell-free culture supernatants.

Overnight cultures of *C. michiganensis* subsp. *michiganensis* inoculated with single colonies and grown in TBY medium were diluted with fresh TBY medium to a final volume of 100 ml with an optical density at 580 nm (OD_{580}) of 0.7 to 0.9. Then, 200 μ l of 10 mM α -tomatine (dissolved in 50 mM Na-citrate, pH 4.0) was added to a final concentration of 0.02 mM α -tomatine. Incubation at 26°C was carried out for 6 h. Bacteria were collected by centrifugation at $4,000 \times g$ for 10 min. The cell pellet was resuspended in 2 ml of PS-buffer (Na_2HPO_4 at 7 g/liter, NaCl at 5 g/liter, KH_2PO_4 at 3 g/liter, pH 7.0).

The resuspended bacterial pellets were filled into a prechilled French press cylinder (AMINCO, Urbana, IL, U.S.A.) and mechanically disrupted by a pressure of approximately 17,000 psi. The procedure was repeated twice with the obtained lysate, followed by centrifugation for 30 min at $10,000 \times g$ to remove cell debris. The protein concentration of the cleared lysate was determined with the BCA Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.) using bovine serum albumin as standard.

Cell-free supernatants were obtained by centrifugation at $4,000 \times g$ for 10 min. The supernatants were transferred into dialysis tubes (10,000-Da cutoff) and dialyzed overnight against solid PEG 40,000 (polyethylene glycol) (Serva, Heidelberg, Germany) at 4°C. Remaining PEG was removed by washing the dialysis tubes with distilled water. The PEG-concentrated samples then were dialyzed against distilled water for 1 to 2 h at 4°C. Up to 10-fold concentration was achieved. The protein concentration was determined with the BCA Protein Assay Reagent (Pierce) using bovine serum albumin as standard. Supernatants were stored at -20°C until used.

TLC analysis of tomatinase reaction products.

C. michiganensis subsp. *michiganensis* crude protein extracts and supernatants were tested for α -tomatine degradation using the method of Lairini and associates (1996) with some modifications. A reaction mixture (200 μ l) containing 1 mM α -tomatine, 20 mM Na-acetate, pH 5.5, 1 mM dithiothreitol (DTT), and 500 μ g of isolated protein extract was incubated at 28°C for 22 h. After incubation, the reaction mixture was vacuum dried, resuspended in 80 μ l of methanol, and 20 μ l each were separated on silica gel 60 F₂₅₄ TLC plates (Merck, Darmstadt, Germany). The plates were developed in a solvent system consisting of ethyl acetate, methanol, acetic acid, and water (30:20:10:1, by volume). Spots were visualized after spraying with 50% H_2SO_4 and heating to 110°C for 10 min. Reaction products were identified by cocomatography with standards (α -tomatine or tomatidine; Sigma, Deisenhofen, Germany) treated the same way. α -Tomatine appeared as a black spot near the bottom line, whereas tomatidine was localized as a dark green spot near the solvent front.

Toxicity test.

TBY soft agar (3 ml, 6% agarose) was mixed with 5 μ l of *C. michiganensis* subsp. *michiganensis* strains (OD_{580} of approximately 6) and applied evenly as a top layer on TBY agar plates. Sterilized paper disks with a diameter of 6 mm containing defined amounts of α -tomatine (resolved in Na-citrate, 50 mM, pH 4.0) were carefully applied on the top layer and incubated for 3 days at 26°C. As negative control, paper disks containing only Na-citrate (50 mM, pH 4.0) without α -tomatine were used. Defined halos around the paper disks showed inhibition of growth by α -tomatine.

Virulence assays in planta.

Two-week-old tomato plants (*L. esculentum* cv. Moneymaker) were infected by dipping the roots into freshly made bacterial suspensions (OD_{580} of 8 to 10) of *Clavibacter* strains for 15 min to enable infection by small vascular lesions through the root. Afterward, the infected plants were planted into sterilized earth and incubated at 25°C for 16 h (12,000 lux) and 19°C for 8 h in the dark with a relative humidity of 40 to 50% in a growth chamber. Each experimental group consisted of 32 plants. The virulent phenotype of *Clavibacter* strains was estimated by examining infected plants for wilting symptoms. Over a period of up to 28 days, plants were examined daily for the development of wilting symptoms (i.e., the occurrence of leaf curling). To get a more quantitative estimation of virulence, the wilting index, defined as the number of days required until 50% of the plants showed wilting symptoms, was determined (Meletzus et al. 1993). Finally, the titer of *Clavibacter* strains per gram of plant material after 28 days of incubation was determined. The plants were cut 1 cm above the ground and homogenized in a sterilized mortar under liquid N_2 . Serial dilutions of the homogenate were plated on TBY plates. Colonies were scored after incubation at 26°C for 5 days.

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