The Putative Secreted Serine Protease Chp-7 Is Required for Full Virulence and Induction of a Nonhost Hypersensitive Response by *Clavibacter michiganensis* subsp. *sepedonicus*

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Molecular biological studies on *Clavibacter michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot of potato, have gained greater feasibility due to the recent availability of whole genomic sequences and genetic tools for related taxa. Here, we describe the first report of construction and characterization of a transposon (Tn) mutant library of *C. michiganensis* subsp. *sepedonicus* sp. strain R10. Since virulence of R10 in potato has been shown previously to be associated with elicitation of a nonhost hypersensitive response (HR), the mutant library was screened initially for loss of HR in tobacco. The screen identified two HR-negative mutants containing Tn insertions within the same gene, CMS2989 (*chp-7*), although at distinct locations. *chp-7* is one of 11 *pat-1* homologs in *C. michiganensis* subsp. *sepedonicus*. HR-negative mutants of R10 multiplied to the same extent as wild type in planta but were less virulent in potato. Complementation with *chp-7* restored virulence as well as the HR phenotype. Together, these findings demonstrate a role for *chp-7* in *C. michiganensis* subsp. *sepedonicus*–plant interactions.

Clavibacter michiganensis subsp. *sepedonicus* is a gram-positive phytopathogenic bacterium and one of five subspecies of the actinobacterial plant-associated species *Clavibacter michiganensis* (Davis et al. 1984; Holt et al. 1994). All known *C. michiganensis* subspecies impact agriculturally important crops such as tomato, potato, and alfalfa, have a limited host

range, and most are vascular pathogens causing wilt as the main symptom (Eichenlaub et al. 2006). *C. michiganensis* subsp. *sepedonicus* is the causal agent of bacterial ring rot of potato, an economically significant plant disease, and, for this reason, is subject to strict national and international quarantines. *C. michiganensis* subsp. *sepedonicus* is tightly plant-associated; it does not survive long in soil or as an epiphyte. The pathogen can establish latent (symptomless) infections as an endophyte for several plant generations until unknown factors lead to symptom outbreaks. Currently, cultivated potato is regarded as the sole host plant of *C. michiganensis* subsp. *sepedonicus*, although effective colonization and symptomatic infection has been reported to occur in *Solanum nigrum* (van der Wolf et al. 2005). Eggplant (*Solanum melongena*) and tomato (*Lycopersicon esculentum*) can be successfully infected in the laboratory and commonly serve as indicator plants in ring-rot testing (Elphinstone 2004).

Currently, two factors are known to be associated with virulence of *C. michiganensis* subsp. *sepedonicus*, cellulolytic activity and ability to induce a nonhost hypersensitive response (HR) (Heath 2000). A secreted cellulase, CelA, is known to be important for symptom development (Laine et al. 2000) but not involved in host colonization. In contrast, pathogenicity and ability to grow in planta correlate with the ability of *C. michiganensis* subsp. *sepedonicus* strains to induce an HR on the nonhost plant tobacco (*Nicotiana tabacum*) (Nissinen et al. 1997). Strains that are unable to induce an HR are nonpathogenic and unable to multiply in planta. The HR phenotype is associated with one or more proteins secreted by the pathogen, but the identity and mode of action of these proteins in disease development are not known.

C. michiganensis subsp. *sepedonicus* and other actinobacterial plant pathogens are still relatively poorly characterized, mostly due to their slow growth rates and the lack of suitable molecular tools, which have made them highly challenging research subjects. However, knowledge on virulence strategies of plant-associated actinobacteria has expanded rapidly in the last several years, since genomic sequences of two subspecies of *C. michiganensis* (subsp. *michiganensis* [Gartemann et al. 2008] and subsp. *sepedonicus* [Bentley et al. 2008]), and a related actinobacterial plant pathogen, *Leifsonia xyli* subsp. *xyli* (Monteiro-Vitorello et al. 2004) became available. Further, development of a transposon (Tn) mutagenesis system

This paper is dedicated to the memory of Dennis L. Knudson, who sadly passed away during its preparation.

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for *C. michiganensis* (Kirchner et al. 2001) combined with genomic sequence information has enabled large-scale studies on these pathogens.

In this paper, we describe construction and characterization of a Tn mutant library of *C. michiganensis* subsp. *sepedonicus* and identify a role in virulence and HR induction of a putative secreted protease from the Pat-1 family (Burger et al. 2005).

RESULTS

Tn library construction and characterization.

A mutant library of *C. michiganensis* subsp. *sepedonicus* sp. strain R10 was constructed, using the arthrobacterial Tn1409Cβ (Gartemann and Eichenlaub 2001) with slight modification of the mutagenesis protocol described by Kirchner and associates (2001). Most *C. michiganensis* subsp. *sepedonicus* strains, including the type strain ATCC33113, are highly mucoid, making cell preparations and isolation of individual colonies on agar plates very difficult. Thus, we chose to use the previously characterized wild-type strain R10, which is highly virulent but nonmucoid (Nissinen 2000). As *C. michiganensis* subsp. *sepedonicus* has been reported to be a highly homogenous subspecies (Brown et al. 2001; Louws et al. 1998), we anticipated limited sequence divergence between R10 and the sequenced strain ATCC33113 (Bentley et al. 2008). Optimal Tn mutagenesis of *C. michiganensis* subsp. *sepedonicus* was achieved by optimizing conditions for preparation of electrocompetent cells and parameters for electroporation (details below). Unlike in *C. michiganensis* subsp. *michiganensis* (Kirchner et al. 2001), we found no apparent difference in transformation rates, whether methylated (from *Escherichia coli* DH5α) or non-methylated DNA (from GM2163) was used. Maximal transformation rates were in the range of 2.5×10^4 CFU per 1 μg of DNA. In total, 30 electroporations yielded a library of 2,100 Tn mutants. The individual mutants were regrown and stored for later use in multititer plates containing YGM (De Boer and Copeman 1980) and 10% glycerol.

To evaluate the effectiveness of Tn1409Cβ for mutagenesis of R10, 408 junction sites of R10 Tn mutants were sequenced. The analyzed data set was limited to 275 Tn mutants for which high-quality sequence reads were obtained. Insertions were localized within insertion (IS) elements and other repeated sequences, coding sequences (CDS), and intergenic sequences. Insertions into repeated sequences were eliminated from the data set, as their location could not be assigned unequivocally. The distribution of the remaining 221 insertions is summarized in Table 1. Tn insertions were distributed throughout the chromosome and within the two plasmids pCSL1 and pCS1. Insertions into pCS1 and pCSL1 were more frequent than insertions into the chromosome. Only 1% of the 3,058 CDS present in the reference strain ATCC33113

were disrupted in strain R10. In contrast, Tn1409Cβ inserted into 27 and 20% of the CDS in pCS1 and pCSL1, respectively. Many of the insertions (52%) were located within relatively low GC regions of the genome or of specific genes. This bias was especially evident in the chromosomal mutations, in which 77% of the inserts were in low-GC regions. Some regions of the genome contained multiple Tn1409Cβ insertions. For example, 55% of the 89 mutations in pCS1 were located in only 18 CDS.

Although the overall percentage of mutants carrying mutations within annotated genes was low, several interesting mutants were identified (Supplementary Table 1). Tn insertions were identified at or very near the *pat-1* homologs on pCS1 (CDS = pCS0022, pCS0037) and on pCSL1 (CDS = pCSL0117). Mutant L11E, (=Cms.R10.YB4) contained Tn1409Cβ within CMS2989, one of the eight chromosomal *pat-1* homologs discovered in *C. michiganensis* subsp. *sepedonicus* (Bentley et al. 2008). Insertions directly into *celA* (pCS0061) were also identified.

Isolation and characterization of HR negative mutants.

As the HR-negative phenotype has been associated with reduced virulence of *C. michiganensis* subsp. *sepedonicus*, we screened the Tn mutant library for induction of HR in *N. tabacum* cv. Samsun, with the goal of isolating HR-negative mutants. After a preliminary screening round, we identified 20 mutants with a severely weakened or negative HR phenotype. Of these 20 mutants, two consistently HR-negative mutants, O10A and L11E, were chosen for further analysis. Both of these mutants grew normally in rich and in YGM (De Boer and Copeman 1980) and were cellulase-positive in plate assays (results not shown) but failed consistently to induce HR in tobacco (Fig. 1).

Tn insertion sites of L11E and O10A were resolved by arbitrary polymerase chain reaction (PCR). Sequencing revealed that both mutants contain insertions of Tn1409Cβ in the same CDS, CMS2989 (Fig. 2A), although at distinct locations (Fig. 2B). Tn1409Cβ insertion sites were 318 and 581 bp downstream from the predicted translation initiation site of *chp-7* for L11E and O10A, respectively. Both mutants have an 8-bp target sequence duplication typical of Tn1409 (Kirchner et al. 2001) at the insertion site (Supplementary Fig. 1) and both contain TAACG as part of the repeat. CMS2989 is also known as *chp-7*, for chromosomal homology of Pat-1 (Bentley et al. 2008), and the name *chp-7* will be used in this paper.

The affected gene, *chp-7*, is a member of a protein family homologous to the pathogenicity determinant and putative serine protease *pat-1* of *C. michiganensis* subsp. *michiganensis* (Burger et al. 2005; Dreier et al. 1997). *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus* contain multiple homologs of *pat-1* with varying similarities

Table 1. Distribution of Tn1409Cβ insertions in *Clavibacter michiganensis* subsp. *sepedonicus* sp. strain R10

Category	Chromosome	pCS1	pCSL1
Number of insertions assigned unambiguously	84	89	48
Number in coding sequences (CDS)	40	18	23
Number with multiple insertions	9	10	8
Number with insertions in low-GC and atypical sequences	25	11	5
Number in intergenic sequences	33	39	12
Number of low-GC and atypical sequences	31	23	2
Total number of insertions (intergenic, CDS, and multiples) in low-GC and atypical sequences	65	39	12
Percent of total assigned insertions located within low-GC and atypical sequences	77	44	25
Percent of total insertions assigned to CDSs	30	56	75
Total number of CDS in replicon ^a	3,058	89	117
Percent CDS disrupted	1	27	20
Percent CDS disrupted in low-GC sequences	63	58	21

^a Bentley et al. 2008.

(Bentley et al. 2008; Gartemann et al. 2008). *chp-7* has highest similarity (86% amino acid identity) to PCS0037, a Pat-1 homolog in the circular plasmid pCS1 of *C. michiganensis* subsp. *sepedonicus* and to Pat-1 family members *pat-1* (YP_001220724) and *phpA* of *C. michiganensis* subsp. *michiganensis* (77 and 71% identity, respectively). In addition to *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus*, homologs of *chp-7* are also present in *Leifsonia xyli* subsp. *xyli* (YP_063162), in *Xanthomonas oryzae* pv. *oryzae* strains KACC10331 (AAW74240), PX099A (ACD60846), and MAFF311018 (YP_449930), in *Acidovorax avenae* pv. *citrulli* (ABM33827.1), and in *X. campestris* pv. *vesicatoria* 85-10 (CAJ26155.1 and CAJ26156.1, *xcv4424* and

xcv4425, respectively). The homologous protein in *X. campestris* pv. *vesicatoria* seems to be inactivated by frameshift, as two successive open reading frames (ORF), *xcv4424* and *xcv4425*, show clear similarity to N-terminal and C-terminal parts of Chp-7, respectively (Supplementary Fig. 2).

Chp-7 has a predicted molecular mass of 29.9 kDa and contains a signal peptide sequence of 35 amino acids (SignalP-hidden Markov model probability of 1.000), with a predicted mature protein of 26.5 kDa. Like other Pat-1 family members, Chp-7 contains two consensus motifs of serine proteases at position 94 (TAKHC) and at position 226 (ATQRGDSGG), the latter including the predicted catalytic serine. Additionally, Chp-7 has a putative membrane-anchoring motif, LPGSGA, close to its C-terminus, present only in certain Pat-1 family members (Gartemann et al. 2008). As Chp-7 is truncated at H106 in mutant L11E and at Y194 in O10A, both mutants lack the protease catalytic site as well as the membrane-anchoring site and are, thus, unlikely to have any protease activity or proper localization (Fig. 2B). Both mutants could putatively produce truncated Chp-7 proteins, with the intact N-terminus of Chp-7 followed by three (in mutant O10A) or ten (in L11E) Tn1409C β -derived amino acids followed by a stop codon in frame.

chp7 resides in a region of the *C. michiganensis* subsp. *sepedonicus* genome with a low percentage of GC (Bentley et al. 2008); the percent GC of *chp-7* is only 57%, remarkably lower than the average of 73% in the ATCC33113 reference chromosome. *chp-7* is located immediately downstream of an IS element (Bentley et al. 2008) and is followed by a putative protein and another *pat-1* family member, CMS2991. As *chp-7* and the adjacent genes are transcribed in opposite directions, *chp-7* is very likely a single-gene operon (Fig. 2A).

Analysis of insertion site of Tn1409C β by PCR and Southern hybridization.

The insertion site and orientation of Tn1409C β in *chp-7* in both mutants was checked by PCR with primer pairs TnpAOut-Pat1F and TnpAOut-Pat1R, in which TnpAOut is located in the *tnpA* region in Tn1409C β and primers Pat1F and Pat1R at the 5' and 3' ends of *chp-7*, respectively. As only primer pair TnpAOut-Pat1F yielded PCR product in both mutants, we concluded that the Tn was inserted in the same orientation in both mutants, and the insertion sites were 325 and 588 bp from the predicted translation initiation site for mutants L11E and O10A, respectively.

Fig. 1. Wild-type strain R10 and mutants L11E and O10A assayed for induction of hypersensitive response on tobacco (*Nicotiana tabacum* cv. Samsun). Bacterial suspensions in 50 mM KPi (pH 7.0) were infiltrated into leaf intercellular spaces with a sterile syringe. Photo was taken 48 h postinoculation. **A**, Wild-type strain R10, **B**, buffer control, **C**, mutant O10A, and **D**, mutant L11E.

Fig. 2. The *chp-7* (CMS2989) **A**, genomic neighborhood and **B**, predicted translation product, with main features and Tn1409C β insertion sites indicated. **A**, Hatched coding sequences are *pat-1* family members; **B**, Tn1409C β insertion sites for respective mutants are marked with black lollipops. Also marked are a putative signal peptide processing site (AQAV) with predicted cleavage between A and V, consensus motifs of the trypsin family of serine proteases (RYVATAKHC and GDSGG), the latter containing the active site serine, and putative membrane-anchoring motif (LPGSGA). Numbers refer to amino-acid residues in protein.

The presence and single site insertion of Tn1409C β in mutants was confirmed by Southern blotting. Total DNA of the wild-type strain R10 and mutants O10A and L11E was hybridized with probes CMS2989int and Tn1409, derived from *chp-7* and Tn1409C β , respectively (details below). Hybridization with probe Tn1409 yielded one band of approximately 800 bp from both mutants, indicating presence of Tn1409C β in both mutant strains (Fig. 3A). When hybridized with probe CMS2989, one band of 3.8 kb was present in R10, while both O10A and L11E showed two separate bands, indicating insertion of Tn1409C β into *chp-7*. An additional CMS2989-specific high-molecular weight band was present in the two mutants as well as in the wild-type strain R10 (Fig. 3B). Based on genome sequence and restriction analysis, this is very likely a highly homologous gene in plasmid pCS1, PCS0037, also known as *php-2*.

L11E and O10A produce truncated *chp-7* transcripts.

We used reverse transcriptase-PCR (RT-PCR) to check, whether mutants L11E and O10A were able to produce (truncated) transcripts of *chp-7*. Three primer pairs were designed with reverse primer upstream of the Tn insertion site in L11E and O10A or downstream of both insertion sites (discussed below). We detected a RT-PCR product in both mutants as well as wild-type strain with a primer pair located upstream of Tn insertion sites in both mutants, indicating that mutants were producing (truncated versions of) *chp7* mRNA (Fig. 4). As expected, no PCR product was detected in RNA samples from mutants with the primer pair corresponding to almost full-size mRNA of *chp-7* (Fig. 4, primer pair 1). Likewise, no product was detected in the L11E sample with a primer pair located downstream of the Tn insertion site in L11E, although a transcript was detected in mutant O10A and wild-type R10A.

Fig. 3. Southern hybridization of *Clavibacter michiganensis* subsp. *sepe-donicus* wild-type strain R10 and mutants L11E and O10A with probes derived from **A**, Tn1409C β and **B**, *chp-7*. Total DNA of wild-type strain R10 and mutants L11E and O10A was isolated, digested with *Bam*H1, and resolved by electrophoresis in 1% agarose gels. Tn1409C β contains two sites for *Bam*H1 (Kirchner et al. 2001). **A**, Tn1409C β -specific signal is present in both mutants but absent from wild-type R10, indicating presence of Tn1409C β in both mutant strains. **B**, One *chp-7*-specific band of 3.8 kb is present in R10. Both O10A and L11E samples contain two separate bands, indicating insertion of Tn1409C β (and subsequent introduction of additional *Bam*H1 cleavage sites) into *chp-7*. The high molecular-weight band present in all three samples and denoted with an asterisk is very likely the highly homologous *pat-1* family member PCS0037, present in plasmid pCS1. Molecular size is marked in kilobases (kb).

Secretomes of *chp-7* mutants do not differ significantly from wild type in vitro.

Based on the presence of a signal peptide sequence, Chp-7 is highly likely to be a secreted protein. As a putative serine protease, it could potentially be involved in protein processing. To determine whether mutations in *chp-7* have a major pleiotrophic effect on other R10 secreted proteins, the secretomes of R10, L11E, and O10A were compared. R10 and mutants L11E and O10A were grown in liquid cultures under conditions known to support production of HR-inducing proteins (Nissinen et al. 1997). After 36 h, supernatants were harvested and concentrated for extracellular proteins, and proteins were resolved by two dimensionnal polyacrylamide gel electrophoresis. The experiment was repeated four times. No drastic or consistent differences were detected in the secretomes of mutant strains as compared with wild type (results not shown).

L11E and O10A are reduced in virulence but are not impaired in their ability to multiply in planta.

We tested the ability of L11E and O10A to induce disease symptoms in eggplant (*Solanum melongena* cv. Black Beauty). After 14 days, disease symptoms were clearly visible in plants infected with wild-type strain R10; plants showed chlorosis, wilting, and stunting. Symptoms were significantly reduced in plants infected with mutant L11E. Plants infected with mutant O10A showed slightly reduced symptoms but were not statistically different from those of R10 (Fig. 5A). The virulence assay was repeated four times with similar results. Severity of disease symptoms produced by mutant L11E was consistently lower than those by R10, but, in three cases out of four, mutant O10A exhibited slightly less virulence that was statistically indistinguishable from wild type. Despite reduced virulence, both O10A and L11E consistently established population densities similar to R10 in planta (Fig. 5B).

The virulence of L11E and O10A was also tested in *S. tuberosum* cv. Superior, which is highly susceptible to bacterial ring rot. Ten weeks after infection, nine of 10 plants inoculated with R10 exhibited severe disease symptoms, with an average disease severity of 76% (Fig. 6). Both mutants were clearly impaired in inducing both disease symptoms (chlorosis, wilting, and necrosis) and size reduction. Again, the difference in

Fig. 4. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *chp-7* transcripts in wild-type strain R10 and mutants L11E and O10A. We used RT-PCR to detect *chp-7* transcripts in R10, L11E, and O10A. Three primer pairs were designed, corresponding either to nearly full-size transcript (primer pair 1) or with reverse primer upstream of the transposon insertion site in O10A (primer pair 2) or L11E (primer pair 3). No PCR product was detected in RNA samples from either of the mutants with primer pair 1. However, a RT-PCR product was detectable in a mutant O10A sample with primer pair 2 and in both mutant samples (as well as in a wild-type strain R10 sample) with primer pair 3, indicating that mutants were producing truncated versions of *chp7* mRNA. DNAase-treated RNA prior to reverse transcriptase treatment was used as a control. In each experiment, some background was visible, indicating residual DNA in the samples. However, the background was in different samples in different runs and, for each strain, we could observe clear positive RT-PCR results without background. The RT-PCR was repeated four times.

virulence was not reflected in population densities. Although in planta population densities of mutants were slightly lower (3.2 and 2.2×10^9 CFU per gram of plant tissue for O10A and L11E, respectively) than R10 (4.3×10^9 CFU/g), the differences were not statistically significant.

Complementation of L11E with *chp-7* restores HR and full virulence.

For complementation of virulence and HR, the coding region and the predicted promoter area of *chp-7* was cloned into an *E. coli*–*Clavibacter* shuttle vector, pHN216 (Laine et al. 1996). The resulting construct pHNPat1Cms was used to transform L11E, the mutant with the more profound virulence phenotype. Three individual transformants of L11E carrying pHNPat1Cms were tested for restoration of HR and virulence. When assayed for induction of HR in tobacco, all transformants carrying pHNPat1Cms induced an HR similar to R10, whereas L11E and L11E transformed with cloning vector pHN216 did not (Fig. 7A). Likewise, when tested for virulence in eggplants, the complemented L11E transformants all induced disease symptoms statistically indistinguishable from R10, confirming that *chp-7* is involved in symptom development (Fig. 7B and C).

Fig. 5. Virulence and population densities in planta of *Clavibacter michiganensis* subsp. *sepedonicus* wild-type strain R10 and mutants O10A and L11E in eggplant. Eggplant stems were inoculated with bacterial suspensions prepared from individual strains, and disease index and bacterial numbers were determined 14 days after inoculation. The data presented represents mean values and standard error from a typical experiment analyzed by analysis of variance followed by Tukey's honestly significant differences test. **A**, Disease severity (%) in eggplants infected with R10, O10A, and L11E. Values marked with the same letter do not differ significantly ($P = 0.05$). **B**, Population densities of R10, O10A, and L11E expressed as mean log CFU per gram of plant tissue, as determined by dilution plating. Values marked with the same letter do not differ significantly ($P = 0.05$).

DISCUSSION

In this paper, we describe the optimization of a Tn mutagenesis system and construction of a Tn mutant library with Tn1409C β (Kirchner et al. 2001) for *C. michiganensis* subsp. *sepedonicus* sp. strain R10. Screening of the library for mutants affected in ability to elicit an HR in a nonhost plant and disease in eggplant, a commonly used indicator plant used in bacterial ring rot diagnosis, yielded several mutants for further study. Further, we demonstrate a role for Chp-7, one of several putative secreted serine proteases of the Pat-1 family, in *C. michiganensis* subsp. *sepedonicus*–plant interactions.

The Tn mutagenesis system developed for *C. michiganensis* subsp. *michiganensis* by Kirchner and associates (2001) was functional in *C. michiganensis* subsp. *sepedonicus* and produced mutants for plant-microbial interaction studies. As reported previously, the glycine concentration used for producing competent cells was critical for optimal transformation. Even with optimization, transformation efficiencies of *C. michiganensis* subsp. *sepedonicus* were much lower (10- to 1,000-fold) than those reported for *C. michiganensis* subsp. *michiganensis*. Multiple electroporations were necessary to obtain a library of 2,100 R10 mutants. Although the R10 mutant library described here represents only a small portion of what would be needed for saturation of the genome, it has significance as the first large scale set of *C. michiganensis* subsp. *sepedonicus* mutants ever characterized in planta.

Tn1409C β , while clearly useful, has limitations as a mutagenesis tool for investigating the molecular biology of *C. michiganensis* subsp. *sepedonicus*. An ideal transposition system would insert randomly within genes of the target organism. However, based on sequence reads of 10% of the R10 mutant library, Tn1409C β did not insert randomly into *C. michiganensis* subsp. *sepedonicus*. Transposition into IS elements was common; nearly 20% of the 275 mutants evaluated contained insertions within IS elements. Tn1409C β would likely have greater utility for genomes that have fewer IS elements; *C. michiganensis* subsp. *sepedonicus* contains a large number (106) of IS elements, whereas IS elements in *C. michiganensis* subsp. *michiganensis* are scarce (Bentley et al 2008; Gartemann et al 2008). There also appears to be a preference of transpositions into plasmids and regions of relatively lower GC content.

Fig. 6. Virulence of *Clavibacter michiganensis* subsp. *sepedonicus* wild-type strain R10 and mutants L11E and O10A in *Solanum tuberosum* cv. Superior. Roots of micropropagated potato plants were inoculated with bacterial suspensions prepared from individual strains and scored for disease symptoms 77 days after inoculation. The data presented represent mean values and standard error, analyzed by analysis of variance followed by Tukey's honestly significant differences test. Values denoted with the same letter do not differ significantly ($P = 0.05$).

Nonetheless, the apparent bias of Tn1409C β for low-GC sequences was advantageous, as many *pat-1* homologs, like CMS2989, are located within genomic islands characterized by lower GC.

Pathogenicity assays of *C. michiganensis* subsp. *sepedonicus* are time consuming and require several plants per test strain. Thus, based on previous work demonstrating a correlation between pathogenicity and HR-production (Nissinen et al. 1997), library screening was expedited by screening first for loss of HR induction in tobacco. This approach identified two HR-negative mutants containing Tn insertions within the same gene, CMS2989 (*chp-7*), although at distinct locations. Further screening demonstrated these mutants were also deficient in virulence. These findings are significant for three reasons. First, the HR phenotype was effective for informing studies on pathogenicity

of *C. michiganensis* subsp. *sepedonicus*. Second, redundancy of *pat-1* homologs within the genome of *C. michiganensis* subsp. *sepedonicus* did not prevent the determination that a specific member of this family contributes to *C. michiganensis* subsp. *sepedonicus*–plant interactions. Thirdly, all *pat-1* homologs do not contribute equally to HR and disease. This is suggested by the fact that mutants containing Tn insertions in three of the 11 *pat-1* homologs in *C. michiganensis* subsp. *sepedonicus* were not identified as HR-deficient in tobacco.

HR induction has been associated with one or more proteins present in culture filtrates of R10 (Nissinen et al. 1997, 2001). Since *chp-7* is predicted to encode a secreted protein, Chp-7 might be produced in culture filtrates and perhaps be responsible for the HR phenotype of *C. michiganensis* subsp. *sepedonicus* in *N. tabacum*. However, as the constructed library is

Fig. 7. Complementation of hypersensitive response (HR) and virulence of *Clavibacter michiganensis* subsp. *sepedonicus* mutant L11E by *chp-7*. **A**, Induction of HR in tobacco by wild-type strain R10, mutant L11E, and L11E transformed with vector pHN216 or with pHNPat1Cms carrying *chp-7*. Samples 1 to 3, L11E transformed with pHNPat1Cms (L11E1, L11E3 and L11E4); sample 4, buffer control; 5: L11E; 6, L11E transformed with pHN216; and 7, wild-type strain R10. Bacterial suspensions in 50 mM KPi (pH 7.0) were infiltrated into leaf intercellular spaces with a sterile syringe. Photo was taken 24 h postinoculation. **B**, Wild-type strain R10, mutant L11E, and L11E transformed with vector pHN216 (L11EP) or with pHNPat1Cms carrying *chp-7* (L11E1, L11E3 and L11E4). Eggplants stems were inoculated with bacterial suspensions prepared from individual strains, and the disease index determined 14 days after inoculation (dpi). The data presented represent mean values and standard error from 10 plants, analyzed by analysis of variance followed by Tukey's honestly significant differences test. Values denoted with the same letter do not differ significantly ($P = 0.05$). **C**, Disease symptoms induced in eggplants by R10, mutant L11E (L), and L11E transformed with vector pHN216 (LP) or with pHNPat1Cms carrying *chp-7* (L1, L3, and L4) or with sterile phosphate buffer (B). The photograph was taken 14 dpi. The plants presented represent typical results.

not saturated, we cannot conclude that Chp-7 is the sole HR-associated protein produced by R10. Additionally, our attempts to express *chp-7* in *E. coli* were unsuccessful, as Chp-7 has a deleterious effect on *E. coli* M15 (data not shown). Thus, we cannot exclude the possibility that Chp-7 functions in the processing of other secreted proteins, although we did not detect any major differences in secreted protein profiles between wild type and the mutants.

In subspecies *michiganensis*, induction of HR is associated with another member of the Pat-1 protein family, ChpG (Gartemann et al. 2008). Chp-7 and ChpG are relatively divergent. ChpG is more similar to CMS2909, another Pat-1 homolog located on the chromosome of *C. michiganensis* subsp. *sepedonicus*. However, the HR tests with subsp. *michiganensis* are routinely done in *Mirabilis jalapa* and subsp. *sepedonicus* has been tested in tobacco. Evaluating the HR phenotypes of Chp-7 and ChpG on *M. jalapa* and *N. tabacum*, respectively, might determine whether elicitation is plant specific. Interestingly, unlike mutants L11E and O10A of *C. michiganensis* subsp. *sepedonicus*, ChpG mutants of *C. michiganensis* subsp. *michiganensis* are fully virulent (Gartemann et al. 2008).

In a recent study on in planta expression of putative virulence determinants of *C. michiganensis* subsp. *sepedonicus*, expression levels of *chp-7* were reported to be slightly lower in planta than in liquid NYB medium (Holtmark et al. 2008). However, as the expression levels were based on an infiltrated, detached leaf assay, the test conditions might not have fully mimicked the in planta conditions of natural xylem-inhabiting cells of *C. michiganensis* subsp. *sepedonicus*. Additionally, one or more HR-inducing proteins can be readily isolated from supernatants of *C. michiganensis* subsp. *sepedonicus* cultures grown in liquid media (Nissinen et al. 1997), indicating that HR-associated genes, probably including *chp-7*, are expressed in standard laboratory medium.

Mutants O10A and L11E both contain Tn1409C β insertions within the same gene, CMS2989, which is very likely the only gene in an operon (Fig. 2A). Still, mutant L11E caused consistently milder (statistically significantly different) disease symptoms in eggplant than O10A (Fig. 5A). In the highly susceptible potato cultivar Superior, however, the differences between the two mutants and R10 was clearly evident, measured either by size reduction (data not shown) or disease severity (Fig. 6). The puzzling differences between phenotypes in eggplant and potato might be related to differences in the Tn1409C β insertion sites. RT-PCR demonstrated that both mutants produced truncated versions of *chp7* transcript (Fig. 4). Thus, both mutations can result in translated albeit truncated proteins, but the mutant protein in L11E is missing almost two thirds of the original protein (174 amino acids of 280) whereas O10A is missing only 86 amino acids. Alternatively, it might be that mutant O10A is still able to produce and secrete a truncated protein while L11E cannot. Although both mutations theoretically yield truncated proteins, the resulting predicted protein in O10A contains only three Tn1409C β -derived amino acids, whereas L11E contains 10, which could have a deleterious effect on protein folding or secretion.

Both Chp-7 mutants L11E and O10A were affected in disease induction (Figs. 5A and 6), indicating that Chp-7 has a role in virulence. Since both mutants grew to wild-type levels in eggplant and potato, Chp-7 is clearly not required for multiplication in planta. This resembles the phenotype of the first described Pat-1-deficient mutant of *C. michiganensis* subsp. *michiganensis*. In that case, loss of *pat-1* was associated with drastically reduced disease symptoms in tomato, but in planta bacterial population densities were indistinguishable from the wild-type strain (Dreier et al. 1997). In contrast, another Pat-1 family member of subspecies *michiganensis*, ChpC, is required for multiplication in host plants; the ChpC mutant shows drastically reduced population densities in planta and produces only very weak disease symptoms (Stork et al. 2008). However, unlike Chp-7 mutants, the ChpC mutant as well as Pat-1 mutants of *C. michiganensis* subsp. *michiganensis* remained HR positive, albeit on *M. jalapa*. Thus, the Pat-1 family members seem to be involved in interactions between *C. michiganensis* and plants and different family members seem to have specific roles and divergent phenotypes depending on host-pathogen specificity. *C. michiganensis* subsp. *sepedonicus* has 11 pat-homologs, eight of which are chromosomal, two are on plasmid pCS1, and one—pCSL0117, with high similarity to ChpC of subsp. *michiganensis*—is located on the linear plasmid pCSL1 (Bentley et al. 2008). Two of the chromosomal homologs seem to be inactive (Bentley et al., 2008).

In addition to the actinobacterial species *C. michiganensis* and *Leifsonia xyli*, *pat-1* homologs are present in plant pathogenic γ -Proteobacteria: in three strains of *X. oryzae* pv. *oryzae*, in *Acidovorax avenae* pv. *citrulli*, and (a possibly inactive homolog) in *Xanthomonas campestris* pv. *vesicatoria* (Table 2). In contrast, no homologous genes have been detected in assembled sequences of *X. oryzae* pv. *oryzicola* or in *X. campestris* pv. *campestris*. All Pat-1 family members from subspecies *sepedonicus* and *michiganensis*, as well as the homologs from gram-negative bacteria, are potentially active serine proteases, as the active sites of serine proteases are conserved across all family members. *X. campestris* pv. *vesicatoria* ORF Xcv4424 and Xcv4425, which seem to represent N-terminus and C-terminus ends of an inactivated homolog, also have intact serine protease active sites. All species but those of genus *Clavibacter* seem to contain only one *pat-1* homolog. Thus, the expansion of the *pat-1* family seems to be *C. michiganensis*-specific. Intriguingly, like CMS2989 (encoding Chp-7) and other *pat-1* homologs in *C. michiganensis* (Bentley et al. 2008; Gartemann et al. 2008), the non-*Clavibacter pat-1* family members also seem to be located in genomic areas that show signs of putative mobile or pathogenicity islands; the *pat-1* homologs in *X. oryzae* pv. *oryzae* strains are all located immediately between IS elements or transposase-like genes and the putatively inactivated homolog in *X. campestris* pv. *vesicatoria* 85-11 is situated 1.3 kb upstream of a putative avirulence gene, *avrRxo1*. Similarly, *pat-1* of *L. xyli* pv. *xyli* (Lxx24245) is downstream from a phage-related lysozyme *lysL* and approximately 4 kb upstream from a putative hemagglutinin hemolysin-like protein (Lxx24270) and a phage-related inte-

Table 2. Non-*Clavibacter* members of the Pat1 protein family and their similarity to Chp-7

Organism	GenBank accession, locus tag, gene	Homology/similarity (%)	Probability
<i>Leifsonia xyli</i> subsp. <i>xyli</i> CTCB07	YP_063162.1, Lxx24245, pat1	30/42	1e-23
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> KACC10331	AAW74240	35/48	9e-28
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> PX099A	ACD60846	35/48	7e-28
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i> MAFF311018	YP_449930	35/48	7e-28
<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10	CAJ26155.1, xcv4424	43/57	7e-21
<i>X. campestris</i> pv. <i>vesicatoria</i> 85-10	CAJ26156.1, xcv4425	35/42	0.69
<i>Acidovorax avenae</i> pv. <i>citrulli</i>	ABM33827.1	34/48	2e-27

grase (Lxx24280). The percent GC of *pat-1* of *L. xyli* subsp. *xyli* is also remarkably low, only 56% as compared with the average 67.7% of the genome.

Secreted proteases have been implicated in virulence of several plant-pathogenic bacteria, but exact roles in pathogenesis are generally unknown. *Xanthomonas campestris* pv. *campestris* secretes two extracellular proteases, Prt1 (serine protease of subtilisin-type) and Prt2 (metalloprotease), which are required for full virulence in conditions mimicking natural infection (Dow et al. 1990). Also, in *X. campestris* pv. *oryzae*, protease production has been shown to be needed for virulence, although the identity of the protease is not known (Xu and Gonzalez 1989). In soft rot enterobacteria and in the fire blight pathogen, *Erwinia amylovora*, metalloprotease production has been shown to promote cell-wall maceration or tissue colonization during virulence (Marits et al. 1999; Zhang et al. 1999).

Effector proteins secreted by the type III-secretion (T3S) system are pivotal for virulence of several gram-negative pathogens. Effector proteins enhance virulence in host plants but are often recognized in resistant plant species or cultivars. Several of these effectors have recently been shown to be proteases and to require protease activity for their virulence or HR-inducing ability. AvrPphB of *Pseudomonas syringae* pv. *phaseolicola* is a member of the papain family of serine proteases and depends on an intact catalytic core for its HR-inducing activity (Shao et al. 2002). XopD and several other YopJ family members of effector proteins are proteases that target SUMOylated host proteins (Hotson and Mudgett 2004) and suppress host defense signaling. Perhaps not surprisingly, there is no evidence of a T3S system in any gram-positive plant pathogen. Gram-positive bacteria secrete most proteins through the general secretion (Sec) apparatus. Others are secreted via the twin arginine transport system (McDonough et al. 2005). Some bacteria possess novel mechanisms for protein secretion and translocation of effectors into their hosts, a functional equivalent to the T3S system (Madden et al. 2001; Pallen 2002; Rosch and Caparon 2005). It is presumed but has not yet been demonstrated that members of the Pat-1 family are secreted through a Sec-dependent pathway. Furthermore, it is not known whether any proteins secreted by *Clavibacter* spp. are translocated into plant cells.

Our studies provide insights on the molecular basis for plant-*Clavibacter* interactions leading to HR or disease. *C. mi-*

chiganensis subsp. *sepedonicus* is a highly specialized plant biotrophic necrogen that has evolved to thrive within a relatively narrow ecological niche, that of the potato apoplast. To accomplish this, it likely produces extracellular enzymes capable of degrading plant cell walls and other compounds in the apoplast as sources of nutrients. It must also be able to somehow circumvent or avoid eliciting plant defense responses. Secreted proteins or those proteins attached to the surface of the bacterial cell would be the first point of contact between this pathogen and its host. Evidence presented here with subspecies *sepedonicus* and elsewhere with subsp. *michiganensis* demonstrates that certain *pat-1* homologs are important in determining the outcome of coryneform-plant interactions. The Pat-1 family constitutes a conserved pathway in plant pathogenesis that is distinct from the more well-studied T3S-dependent pathway. Continued study of *pat-1* homologs in *C. michiganensis* is essential for gaining a better understanding of the specific molecular events leading to disease or HR elicitation by this group of largely host-specific plant pathogens. Such studies could also contribute generally to a comprehensive understanding of pathway diversity underlying plant-microbe interactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The bacterial strains and plasmid constructs used in the study are listed in Table 3. Routinely, *C. michiganensis* subsp. *sepedonicus* strains were grown at 23 to 26°C on YPG (0.5% yeast extract, 0.5% bacto peptone, 1.0% glucose, all wt/vol) or, prior to HR tests, on YGM (De Boer and Copeman 1980). All cultures were started from glycerol stocks stored at -20 or -80°C. Media were supplemented with 40 µg of neomycin per milliliter and 2.5 µg of chloramphenicol per milliliter, as necessary. *Escherichia coli* Top10F⁺ (Invitrogen, Carlsbad, CA, U.S.A.) was grown at 37°C in Luria-Bertani medium containing 150 µg of ampicillin per milliliter. All selective plates used for screening positively transformed *E. coli* colonies had 80 µl of 20 mg/ml X-gal and 10 µl of 0.5 M isopropyl-β-D-thiogalactoside (IPTG) spread on the surface.

Plant assays.

Virulence of *C. michiganensis* subsp. *sepedonicus* mutants was evaluated in eggplant and in potato. For eggplant assays, stems of 14- to 18-day-old (first true leaf emerging) eggplants (*Solanum melongena* cv. Black Beauty) were inoculated with bacterial suspensions (approximately 10⁸ CFU/ml) and were rated for disease symptoms 14 days after inoculation (dpi), as described by Nissinen and associates (1997). Eight to twelve plantlets were used per treatment, and each assay was repeated four times. Bacterial population densities in eggplants were determined 14 dpi. Preweighed stem sections were surface sterilized and ground in sterile 50 mM KPi (pH 7.0). Serial dilutions of homogenates were plated onto YPGA (solid YPG medium with 1.5% [wt/vol] agar plates), and bacterial population densities were calculated after 1 week at room temperature (approximately 23°C). *Solanum tuberosum* cv. Superior was used to evaluate the virulence of the mutants in potato, essentially as described by Brown and associates (2002). Roots of 4-week-old micropropagated plantlets were soaked in bacterial suspensions adjusted to an optical density at 620 nm (OD₆₂₀) = 0.2 in 50 mM KPi or in sterile buffer for a negative control. Plantlets were transplanted in soil-peat mix and were grown at 22°C in a growth chamber. Symptoms were evaluated at 77 dpi. Tests for HR on tobacco were performed as described by Nissinen and associates (1997). Routinely, tobacco plants (*Nicotiana tabacum* cv. Samsun) were grown in a greenhouse

Table 3. Bacterial strains and plasmids used in the study

Strain	Relevant genotype and/or phenotype ^a	Reference or source
Bacteria		
<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>		
R10	Wild-type strain, hypersensitive-response-positive, virulent, nonmucoid	Nissinen 2000
L11E	Tn1409Cβ mutant of R10, cmx ^r	This work
O10A	Tn1409Cβ mutant of R10, cmx ^r	This work
L11EpPat-1	L11E transformed with pHNPat1Cms	This work
L11EpPat-3	L11E transformed with pHNPat1Cms	This work
L11EpPat-4	L11E transformed with pHNPat1Cms	This work
Plasmids		
pKGT452Cβ	Vector for delivery of Tn1409Cβ, amp ^r (vector) cmx ^r (Tn)	Gartemann and Eichenlaub 2001
pHN216	<i>Clavibacter-Escherichia coli</i> shuttle vector, neo ^r	Laine et al. 1996
pHNPat1Cms	<i>chp-7</i> and predicted promoter in pHN216, neo ^r	This work

^a cmx^r, amp^r, and neo^r = resistant to chloramphenicol, ampicillin, and neomycin, respectively.

to the six- to eight-leaf stage prior to being transferred to a growth chamber (23°C). Leaves were treated by punching a small hole in the leaf blade with a sterile injection needle, followed by infiltration with a needleless, 1-ml syringe containing bacterial suspensions at OD₆₂₀ = 0.8. Responses were routinely scored 24 h postinoculation (hpi) and, during the Tn mutant library screening, also 48 hpi. Statistical tests involving disease severity and population densities were performed by ANOVA (analysis of variance) followed by Tukey's HSD (honestly significant differences) test.

Construction of Tn mutant library.

A Tn mutant library of highly virulent, nonmucoid wild-type strain R10 was constructed following the Tn-based mutagenesis methods of Kirchner and associates (2001), using arthrobacterial Tn1409Cβ (Gartemann and Eichenlaub 2001). The methods were modified for *C. michiganensis* subsp. *sepedonicus*. To obtain electrocompetent cells, cultures were pre-grown from frozen stocks on YGM plates for 2 days, were re-suspended in YGM liquid medium with 0.2 to 0.4% (wt/vol) glycine to an OD₅₄₀ of 0.05, and were grown at 25°C, with vigorous shaking to an OD₅₄₀ of 0.2 to 0.5. The cells were washed three times in sterile 10% glycerol, were resuspended to an OD₅₄₀ of 20 to 50, and were electroporated with pKGT452Cβ with the electroporation parameters set to 25 μF, 600 ohms, and 12.5 to 15 V/cm. Voltages above 15 V/cm reduced transformation rates.

Characterization of Tn library.

A modified cetyltrimethylammonium bromide purification procedure was used to produce template suitable for direct Big Dye terminator v3.1 cycle sequencing (Big Dye terminator cycle sequencing kit from Applied Biosystems, Foster City, CA, U.S.A.). The left ISN2 or the right ISC2 primer (direction relative the Tn1409Cβ) was used to produce the sequence (Kirchner et al. 2001). The sequencing primer was GGAACCTCACCACTACATAGC for ISC2 and CATGCAG TTGCGCCACTACAC for ISN2. Only-high quality sequence reads with high similarities to reference strain CMS ATCC33113 (blastn scores of less than 1e-100 or GFF2 scores >500 or both) were evaluated further. The sequences were localized within the complete genome sequences of reference strain *C. michiganensis* subsp. *sepedonicus* ATCC33113 (Bentley et al. 2008) and were visualized in Artemis (Parkhill et al. 2000). Sequences for pCS1 (accession number AM849035) and pCSL1 (accession number AM8490367) were obtained from the European Molecular Biology Laboratory-European Bioinformatics Institute website.

Arbitrary PCR.

The arbitrary PCR protocol was modified from O'Toole and Kolter (1998). Total genomic DNA of *C. michiganensis* subsp. *sepedonicus* mutants were isolated as described by Comai and Kosuge (1983), suspended in water and boiled 2 min before PCR. The first-round primers were Tn1409out, unique to the 25-bp right inverted repeats of Tn1409Cβ and ARB1, designed to hybridize to an arbitrary sequence on the chromosome. For the second round, nested primers were used: Tn1409nest, unique to the Tn, and ARB2, identical to the 5' end of the arbitrary primer ARB1. The oligonucleotide sequences of the four primers are: Tn1409out: ACTACACCTCAATTCTGAA, ARB1: GGCCACGCGTCGACTAGTACNNNNNNNNNGA TAT, Tn1409nest: TACACCTCAATTCTGAAGAGCC, and ARB2: GGCCACGCGTCGACTAGTAC. The PCR products were cloned into TOPO-TA cloning vector (Invitrogen Life Technologies, Carlsbad, CA, U.S.A) and were sequenced. The sequences were then localized on the assembled *C. michiganen-*

sis subsp. *sepedonicus* genomic sequence available on the Sanger Center *C. michiganensis* subsp. *sepedonicus* website.

PCR to detect and analyze CMS2989.

PCR primers Pat1F (CGT GCA TCG CTC CCT GTG) and Pat1R (TGA CGA CAG GTC CCC CAG) were used to check for the presence of CMS2989 in wild-type strains of *C. michiganensis* subsp. *sepedonicus*. For confirming location and orientation of Tn1409Cβ in mutants L11E and O10A, primers Pat1F, Pat1R, and TnpAout (ATC CAA GGA AAG AAG CTC ATG C) were used in pairs TnpAout-Pat1R and Tnp1-Pat1F. Pat1F and Pat1R were located upstream and downstream, respectively, of the Tn insertion sites in *chp-7* of both mutants, and TnpAout was a 24-bp area located from bp 1,019 to 1,042 of the *tnpA* region in Tn1409Cβ (Gartemann and Eichenlaub 2001).

Southern hybridization.

Chromosomal DNA of R10 and mutants L11E and O10A was isolated as described in Comai and Kosuge (1983), and then digested with *Bam*H1 at 37°C for 6 h and resolved by electrophoresis in 1% (wt/vol) agarose gels. For detection of both the Tn1409Cβ and the mutant *chp-7* genes, two groups of samples from the same digested DNA were run on one gel to get identical blots. Southern blotting to nylon membranes was done overnight as described by Sambrook and associates (1989). Probes for detection of *chp-7* (probe CMS2989int, 595 bp) and Tn1409Cβ (probe 1409, 581 bp) were amplified by PCR from R10 and Tn1409Cβ with primer pairs Pat1F-Pat1R (sequences above) and TnpAF-TnpAR, respectively (TnpAF: TGA CGA CAG GTC CCC CAG, TnpAR: CAG TGT CTG TAC CCT GAT CGT GC). The probes were digoxigenin (DIG)-labeled, by the addition of 0.5 μl of DIG-conjugated UTP included in each PCR reaction (50 μl). Hybridization was done overnight following a protocol by Sambrook and associates (1989), modified as follows. The probes were denatured by heating in boiling water for 2 min prior to hybridization. All hybridizations and posthybridization washes were carried out at 68°C in a hybridization oven (MIDI DUAL 14, Hybaid Ltd., Teddington, Middlesex, U.K.). Detection of the hybridization results was done by Chemiluminescence detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

RT-PCR.

We designed three primer pairs with a common forward primer and reverse primers specific for full-size transcript and both of the truncated mutant transcripts as follows:

forward primer

(F1) TCTGTGAGAATCCCACA (binds to nucleotides (nt)

10 to 26 of *chp-7*);

reverse primers

R(wt) AGGTCTCATCTCCTGAAC (binding site gttcagga gatgagacct, 65 nt upstream of predicted translation stop)

R(O) AAATCTCCCGAGCATCTG (binding site GATGC TCGGGAGATTTT 22 nt upstream of the Tn insertion site in O10A) and

R(L) GCGGTCGCTACATATCG (binding site cgaatgtag cgaccgc 41 nt upstream of the Tn insertion site in L11E).

For RNA extraction, the bacteria were cultivated 48 h. The bacteria were harvested by centrifugation, and cells were broken by FastPrep (MP Biomedicals, Illkirch, France). Total RNA was extracted with RNeasy plant mini kit (Qiagen, Hilden, Germany). RNA (1 μg) was treated with 2 U DNase (Promega, Madison, WI, U.S.A.) for 3 h at 37°C. The DNase reaction was stopped and RT-PCR was performed by adding 200 ng of random hexamers. The reaction was incubated at 70°C for 10

min and was then snap cooled on ice. The following ingredients were added to each sample: reaction buffer (1×), deoxynucleoside triphosphates (0.5 mM each), dithiothreitol (10 mM), RNasin (20 U, Promega), and reverse transcriptase (400 U, Promega). cDNA synthesis was carried out by incubating first for 10 min at 25°C and then for 90 min at 37°C. The reaction was stopped by incubating for 10 min at 70°C. The cDNA was diluted by the addition of 80 µl of H₂O to each sample, and 3 µl was used as a template for 25-µl PCR reactions. PCR was performed with *Taq* polymerase (Promega), following the instructions of the manufacturer. A Dnase-treated RNA sample was used as a control to detect putative chromosomal DNA contamination.

Complementation of L11E mutant strain with CMS2989.

The *pat1* gene and its promoter site from R10 was amplified with PCR, using primers Pat1CmsF (CGTATCCGATGAGCTGTCCTG) and Pat1CmsR (AAATATTTCACACCATCCGCTGAG) as forward and reverse primers, respectively. The PCR fragment was A-tailed with *Taq* polymerase and was cloned in TOPO-TA cloning vector (Invitrogen Life Technologies). The insert and pHN216 plasmid vector were cut with *Eco*RI and *Ssp*I and were purified (QIAGEN PCR purification kit) and ligated. The correct structure of the resulting construct, pPat, was verified by PCR and sequencing, and the construct was electroporated into electrocompetent L11E cells. Preparation of electrocompetent cells and the transformation parameters were as described above for construction of the Tn library, with the exception that no glycine was added to growth media. The cloning vector pHN216 was used to construct a vector-control strain. Transformants were plated onto YPGA supplemented with 40 µg of neomycin per milliliter. The presence of appropriate plasmids in transformants was confirmed by PCR.

Expression of Pat1 in *E. coli*.

pat-1 was amplified from R10 with primer pair Pat1ECF (CCGCCCATGGCAGTCGATCGCA) and Pat1ECR (GCCGCATGGTCCGCGAGTACGT). The forward primer was designed to exclude the leader peptide of CMS2989 and was modified to ensure correct frame and expression in *E. coli*. The stop codon included in the reverse primer omitted the pQE-60-derived histidine tag from the fusion protein. The PCR fragment was cloned in TOPO-TA cloning vector (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and, after propagation in *E. coli* DH5α, was transferred into pQE-60 (Qiagen) with *Nco*I (Fermentas, Vilnius, Lithuania). The correct orientation of the insert in pQE-60 was verified by PCR, and the resulting construct was named pPat1EC. For protein expression, the *E. coli* M15 was chemically transformed with pPat1CmsEC and, for vector control, with pQE-60 as described by Sambrook and associates (1998). Protein production was induced with 0.1 to 1 mM IPTG.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- J. Craig Venter Institute website: www.tigr.org/db.shtml
 European Molecular Biology Laboratory-European Bioinformatics
 Institute: www.ebi.ac.uk/genomes/plasmid.html
 The Sanger Center *C. michiganensis* subsp. *sepedonicus* website:
www.sanger.ac.uk/Projects/C_michiganensis