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A family of serine proteases of *Clavibacter michiganensis* subsp. *michiganensis*: *chpC* plays a role in colonization of the host plant tomato

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SUMMARY

Genes for seven putative serine proteases (ChpA–ChpG) belonging to the trypsin subfamily and homologous to the virulence factor *pat-1* were identified on the chromosome of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) NCPPB382. All proteases have signal peptides indicating export of these proteins. Their putative function is suggested by two motifs and an aspartate residue typical for serine proteases. Furthermore, six cysteine residues are located at conserved positions. The genes are clustered in a chromosomal region of about 50 kb with a significantly lower G + C content than common for *Cmm*. The genes *chpA*, *chpB* and *chpD* are pseudogenes as they contain frame shifts and/or in-frame stop codons. The genes *chpC* and *chpG* were inactivated by the insertion of an antibiotic resistance cassette. The *chpG* mutant was not impaired in virulence. However, *in planta* the titre of the *chpC* mutant was drastically reduced and only weak disease symptoms were observed. Complementation of the *chpC* mutant by the wild-type allele restored full virulence. *ChpC* is the first chromosomal gene of *Cmm* identified so far that affects the interaction of the pathogen with the host plant.

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is a Gram-positive, plant pathogenic actinomycete (family Microbacteriaceae) (Stackebrandt *et al.*, 1997) causing bacterial wilt and canker of tomato plants (*Solanum lycopersicum* L.) (Davis *et al.*, 1984; Strider, 1969). Infection by *Cmm* results in a systemic tracheobacteriosis with an *in planta* titre of 10^9–10^{10} bacteria per gram plant homogenate. The first symptom of disease is unilateral wilting, followed at later stages by canker lesions on the stem (Wallis, 1977). The wild-type strain NCPPB382 carries two plasmids that are essential for virulence, pCM1 and pCM2 (Meletzus *et al.*, 1993). Both plasmids carry a single virulence factor, the gene *celA* coding for an endo-β-1,4-glucanase on plasmid pCM1 (Jahr *et al.*, 2000), and the gene *pat-1* encoding a putative serine protease on plasmid pCM2 (Dreier *et al.*, 1997). Loss of one plasmid, either pCM1 with *celA* or pCM2 with *pat-1*, reduces virulence, i.e. development of disease symptoms is delayed. When both plasmids are lost a complete loss of virulence results and the typical wilting symptoms do not occur, although the bacteria are still able to colonize the host plant. Thus, the loss of the virulence factors converts *Cmm* into a non-virulent endophyte of tomato (Dreier *et al.*, 1997; Gartemann *et al.*, 2003; Jahr *et al.*, 2000; Meletzus *et al.*, 1993). This shows that gene functions required for the colonization of the host plant are encoded by the chromosome.

Hybridization of total DNA of the wild-type strain NCPPB382 against a *pat-1* probe led to the identification of three homologous genes, termed *phpA/B*, carried on plasmid pCM2, and the pseudogene *chpA*, located on the chromosome (Burger *et al.*, 2005). It was shown that these genes are not involved in *Cmm*–tomato interaction. *Clavibacter michiganensis* subsp. *sepedonicus* is the causal agent of bacterial wilt and ring rot of potato and also carries several *pat-1* homologous genes (Holtsmark *et al.*, 2008). Using quantitative reverse transcriptase (RT)-PCR, five of these genes were analysed during infection of potato and in liquid culture. It was shown that during infection three *pat-1* homologous genes were downregulated and two were upregulated, implying an involvement of these genes in the infection process (Holtsmark *et al.*, 2008).

In *Cmm*, analysis of the nucleotide sequence surrounding the *chpA* locus revealed that *chpA* maps in a cluster of six additional genes putatively encoding serine proteases with homology to Pat-1. Due to the relationship of the *chp* genes to the virulence gene *pat-1* we considered it possible that these genes could be involved in the interaction of *Cmm* with the host plant. Here we present the classical genetic approach specifically to inactivate candidate genes that might be involved in the pathogenicity of *Cmm* and to analyse the phenotype of the corresponding mutants in comparison with the respective wild-type reference strain.

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Nucleotide sequence data are to be found at GenBank as accession number AM711867 (complete chromosome).

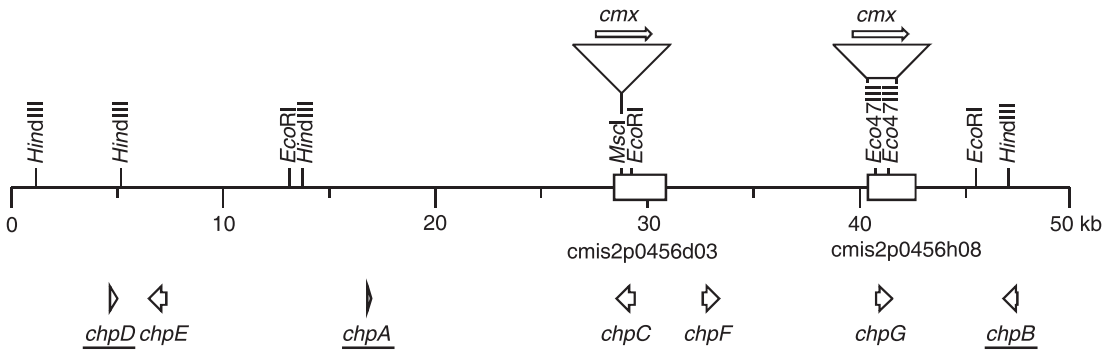

Fig. 1 Physical map of the 50-kb chromosomal region of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) carrying the *chp* genes. Pseudogenes are underlined. Boxes indicate the fragments used to construct plasmids for gene replacement or complementation. The insertion sites of the cassettes carrying the chloramphenicol exporter gene *cmx* are indicated.

RESULTS

Features of the Pat-1 protein family

In a recent publication (Burger *et al.*, 2005) we have reported on the *chpA* gene (chromosomal homology of *pat-1*). *chpA* is a pseudogene, containing internal stop codons as well as two frame shifts. The hypothetical gene product is homologous to Pat-1, indicating that the functional gene could encode a putative serine protease. Analysis of the nucleotide sequence around the *chpA* locus revealed six more *pat-1* homologous genes (*chpB*, *chpC*, *chpD*, *chpE*, *chpF* and *chpG*) in a region of about 50 kb of the chromosome (Fig. 1).

Like *chpA*, the genes *chpB* and *chpD* are also pseudogenes. *ChpB* contains a frame shift 138 nt downstream of the ATG start codon and *chpD* has a frame shift at position 114 downstream of the ATG start codon and an in-frame stop codon at position 547–549. For the multiple alignment of the Chp proteins the reading frames of ChpA, ChpB and ChpD were restored at the appropriate amino acid positions (Fig. 2). All Pat-1 homologues have a putative signal peptide indicating that these proteins are secreted. Two motifs characteristic for serine proteases of the trypsin type ([LIVM][ST]A[STAG]**H**C and [DNSTAGC][GSTAPIMVQH]x(2)G[DE]**S**G[GS][SAPHV][LIVMFYWH][LIVMFYSTANQH]-PROSITE, PDOC00124) are highly conserved. The histidine and serine residues indicated by bold letters in the motifs are those amino acids participating in catalysis. An aspartate as part of the catalytic triad is also conserved. Furthermore, six cysteine residues are located at conserved positions in all members of this protein family except in ChpC, which has only four of the conserved cysteine residues. The functional members of the Pat-1 family have 277–286 amino acid residues and molecular weights of between 29.0 and 35.8 kDa. The G + C content of the genes varies between 51.9 and 65.5 mol% and thus is significantly lower than the average G + C content of 72.6 mol% for *Cmm.* Furthermore, the codon usage deviates from the normal codon usage of *Cmm*. The presence of rare codons as observed for *pat-1* and *chpA* (Burger *et al.*, 2005) is a common feature of all *chp* genes. A putative sortase motif with homology to (LPXTG) (Mazmanian *et al.*, 2001), which may be important for anchoring of the protein to the cell wall, was identified only in Pat-1 and the ChpA pseudogene.

Construction of *chpC* and *chpG* mutants

In order to assess a possible function for the *chp* genes in plant–microbe interaction, *Cmm* NCPPB382 was subjected to targeted homologous recombination for the generation of *chp* mutants. Thus far, we have been able to obtain mutations in the genes *chpC* and *chpG*.

A 1.9-kb *Bsa*AI DNA fragment containing the chloramphenicol resistance gene (*cmx*) from hybrid plasmid pEC70 (Tauch *et al.*, 1998) was inserted into the internal *Msc*I site of the *chpC* gene carried by plasmid cmis2p0456d03, a sequenced plasmid from the shotgun cloning of the genome project (Fig. 1). Both orientations of the *cmx* cassette relative to *chpC* were obtained (pIGCα and pIGCβ). In plasmid cmis2p0456h08, which carries the *chpG* gene (Fig. 1), an internal 640-bp *Eco*47III fragment was replaced by a 1.9-kb *Bsa*AI fragment of pEC70 carrying the *cmx* gene. Both orientations of the *cmx* cassette relative to *chpG* were obtained (pIGGα and pIGGβ). The hybrid plasmids based on the *Escherichia coli* vector pSMART, which cannot replicate in *Cmm*, were introduced into the wild-type strain NCPPB382 by electroporation. Clones in which recombination between the wild-type genes and the inactivated gene on the plasmid had occurred (formation of a cointegrate) were selected with chloramphenicol in the medium. If a second crossover had occured at the other side flanking the *cmx* gene, the intact chromosomal *chp* gene was exchanged by the inactivated *chp* gene.

To detect such knock-out mutants, total DNA of chloramphenicol-resistant clones was isolated, hydrolysed with *Nco*I and *Bam*HI

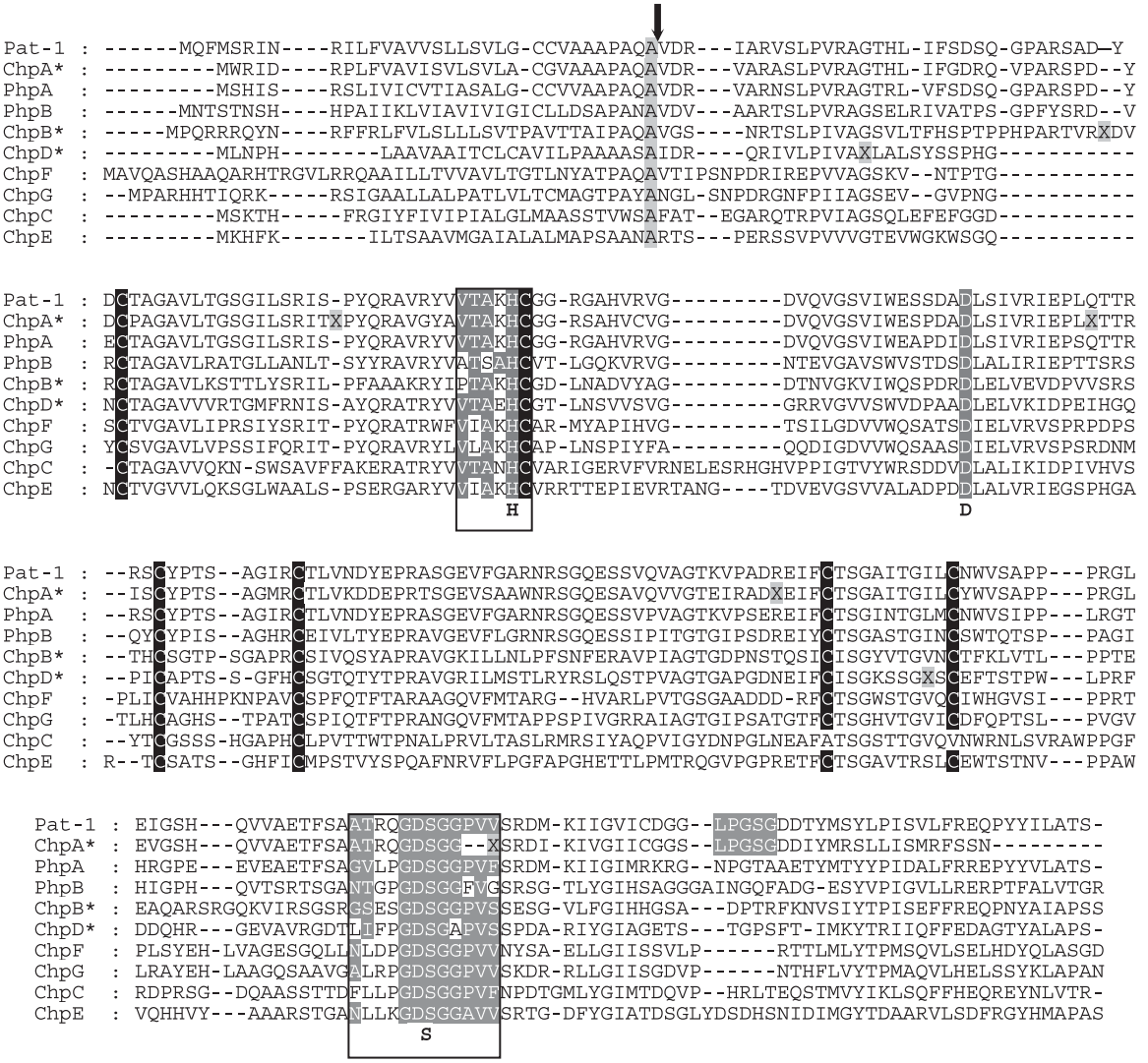

Fig. 2 Multiple alignment of the Pat-1 family members of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Pseudogenes are marked with asteriks. They contain 'X' introduced to restore the reading frame. Conserved cysteines, the conserved aspartate of the catalytic triad, the two serine proteases motifs and the putative sortase motif (LPGSG) are also indicated. The three amino acid residues (H, D, S) of the catalytic triad are exhibited below the alignment. The arrow marks the hypothetical processing site (AQA↓V) although this motif is not conserved in all putative gene products.

and hybridized against a labelled pUC probe which shares homology with pSMART. Clones which gave a signal were interpreted as cointegrates and discarded. Clones which gave no signal with the pUC probe were further analysed by hybridization against *chpC* and *chpG* probes, respectively. DNA from the *chpC* mutant designated CMM101 *chpC*β gave 2.9-kb and 10-kb signals (*Nco*I digestion) or 2.6-kb and 5.9-kb signals (*Bam*HI hydrolysis) against the *chpC* probe, as expected for a double crossover event with the *cmx* cassette integrated in the β-orientation (Fig. 3). *Nco*I-digested total DNA of the *chpG* mutant designated CMM101 *chpG*β gave signals of 1.1 and 2.9 kb in hybridizations against a *cmx* probe (data not shown) and of 1.1, 2.0 and 2.9 kb against the *chpG* probe, showing that the desired inactivation of *chpG* was achieved and that the *cmx* cassette was integrated in the opposite direction relative to *chpG* (Fig. 4).

In addition, *Bam*HI-digested total DNA was hybridized against the *chpG* probe. The hybridizing bands had sizes of 0.6 and 3.4 kb as expected for a double crossover (Fig. 4).

During electroporation of the wild-type strain *Cmm* NCPPB382 we frequently observed loss of plasmid pCM2. Such derivatives, termed CMM101 (they still carry plasmid pCM1), are still virulent and able to colonize tomato plants efficiently.

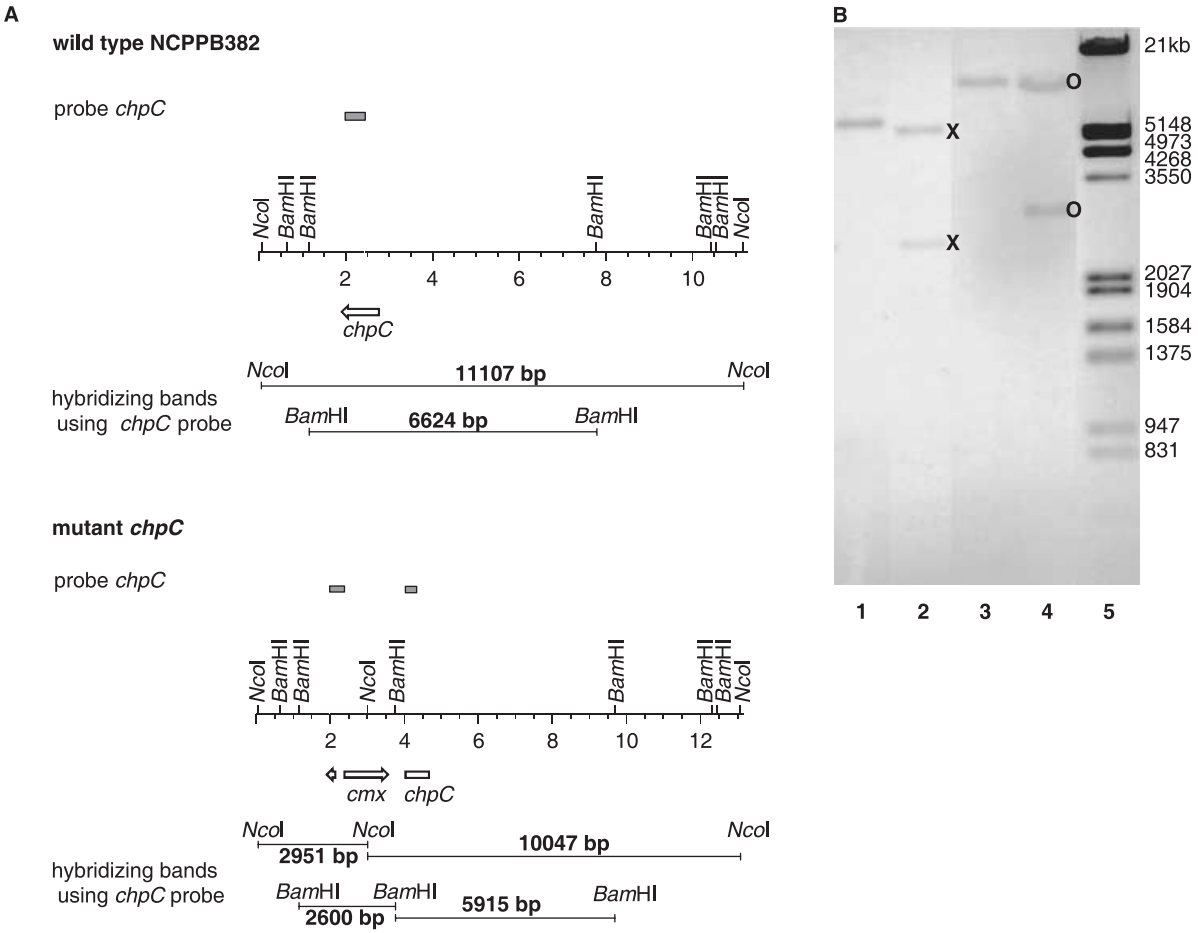

Fig. 3 (A) Physical map of the *chpC* region in the wild-type and in the *chpC* mutant. (B) Southern hybridization against the *chpC* probe. *Bam*HI-digested total DNA of the wild-type (lane1) and the *chpC* mutant (lane 2), *Nco*I-digested total DNA of the wild-type (lane 3) and the *chpC* mutant (lane 4), digoxygenin-11-dUTP-labelled *Eco*RI/*Hin*dIII-digested λ marker (lane 5). The expected signals of 2.9 and 10 kb (*Nco*I digestion, ○) and 2.6 and 5.9 kb (*Bam*HI hydrolysis, ×) were obtained for the *chpC* mutant.

Thus, the *chpC* and the *chpG* mutants were checked for the presence of both plasmids pCM1 and pCM2 via hybridization against specific *celA* (pCM1) and *pat-1* (pCM2) probes. As expected, only *celA* (pCM1) was found while *pat-1* gave no signal, indicating that plasmid pCM2 was lost in the mutants (data not shown). According to their plasmid status, which is identical to the plasmid curing derivative CMM101, carrying only plasmid pCM1 with the pathogenicity factor *celA*, the mutants were designated CMM101 *chpC*β and CMM101 *chpG*β.

Assessment of virulence of *chpC* and *chpG* mutants

In tests for the pathogenic phenotype of mutants CMM101 *chpC*β and CMM101 *chpG* as shown in Fig. 5, 64 tomato plants were infected with one of the mutant strains or with CMM101 as a control. In these experiments CMM101 *chpC*β displayed very weak virulence; only seven of 64 plants showed symptoms with curled and wilting leaves 28 days post-infection (dpi). In contrast to that, infection by the reference strain CMM101 as control, showed curled and wilting leaves in 32 of 64 plants already 15 to 16 dpi (Fig. 5). This comparison shows that virulence of the mutant strain CMM101 *chpC*β is drastically reduced as compared with the reference strain CMM101. The experiment was repeated four times with 32 plants in each experimental group. Although there are variations in the sensitivity of the tomato plants to infection and general vitality of the plants due to seasonal effects, the qualitative results were identical to the example shown in Fig. 5. The wilting indices as shown in Table 1 did vary by 1–2 days in different experiments. However, infection with the *chpC* mutant always resulted in only 2–5 plants with mild disease symptoms out of 32 plants.

When the titre *in planta* was determined, we found that strain CMM101 *chpC*β did not colonize the plants as well as the reference

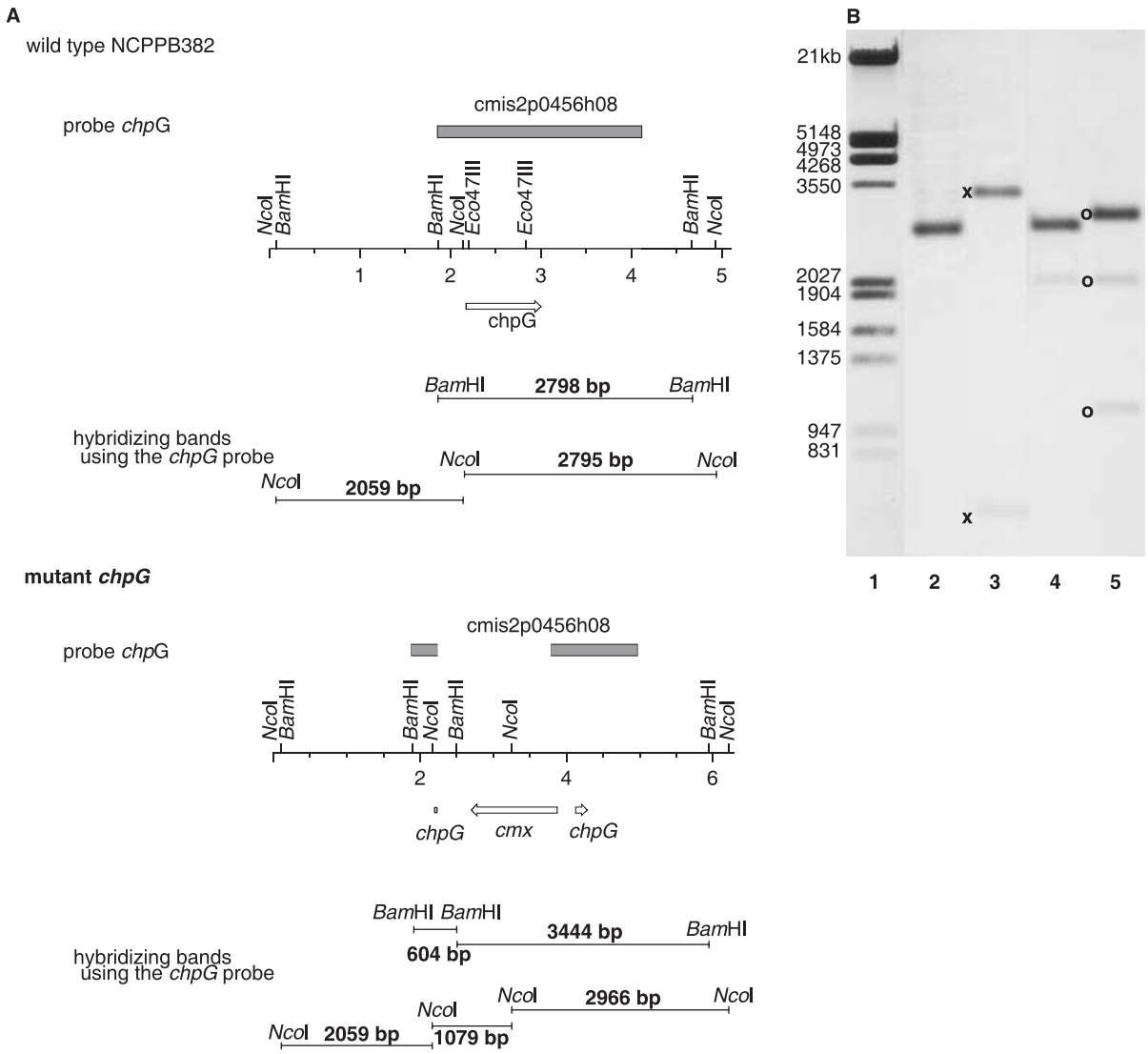

Fig. 4 (A) Physical map of the *chpG* region in the wild-type and in the mutant. (B) Southern hybridization against the *chpG* probe. *Bam*HI-digested total DNA of the wild-type (lane 2) and the mutant (lane 3), *Nco*I-digested total DNA of the wild-type (lane 4) and the mutant (lane 5), digoxygenin-11-dUTP-labelled *Eco*RI/*Hin*dIII-digested λ marker (lane 1). The occurrence of signals of 1.1, 2.0 and 2.9 kb (*Nco*I digestion, ○) and 0.6 and 3.4 kb (*Bam*HI hydrolysis, ×) against the *chpG* probe demonstrated that the desired inactivation of *chpG* was achieved.

strain CMM101 or wild-type *Cmm* NCPPB382. The titre of CMM101*chpC*β was reduced 500-fold to 2×10^7 cfu/g plant homogenate as compared with 1×10^{10} cfu/g plant homogenate for CMM101 (Table 1). Obviously, the *chpC* mutant is not able to colonize the host plant as efficiently as the reference strain. Although the wilt-inducing pathogenicity gene *celA* is present in the mutant strain, the titre *in planta* seems not to be high enough for the development of full virulence. To exclude the possibility that the mutation in *chpC* has an effect on general growth behaviour we determined the generation times in different media. The generation times of the *chpC* and the *chpG* mutants compared with CMM101 did not differ and were 2.5 h in rich medium and 4 h in minimal medium, respectively.

In contrast to CMM101*chpC*β, the *chpG* mutant CMM101*chpG*β was not affected in virulence and colonization of the host plant. When CMM101*chpG*β was compared with the control strain CMM101 the virulence assays and titre *in planta* indicated no significant difference (Fig. 5, Table 1). However, when leaves of the non-host plant *Mirabilis jalapa* were infiltrated with cell suspensions of the *chpC* and the *chpG* mutants we observed no induction of a hypersensitive reaction (HR) for the *chpG* mutant (data not shown). This indicates that the putative serine

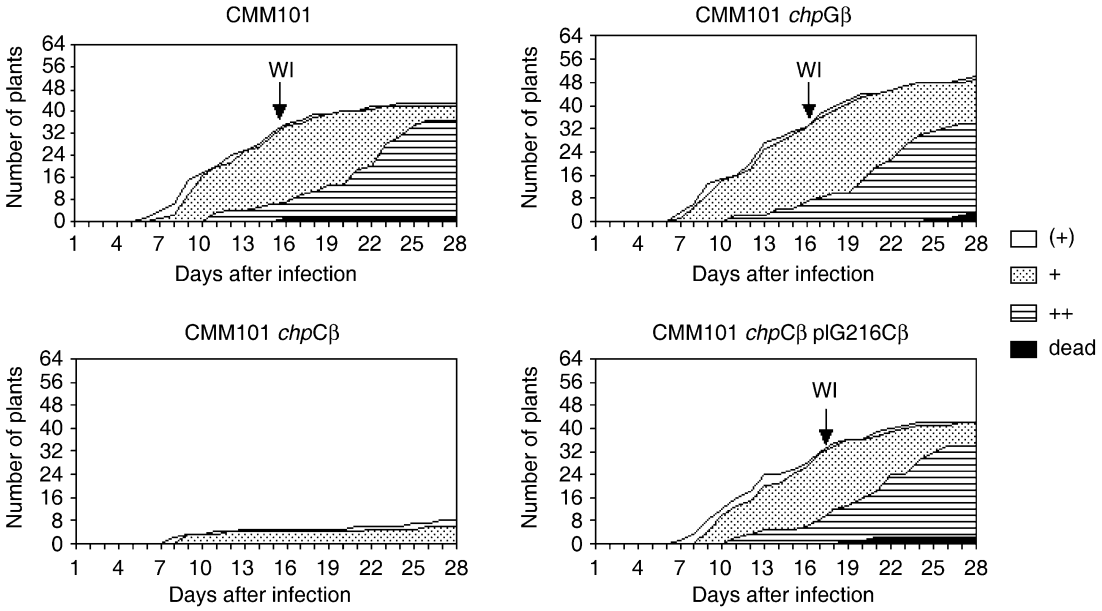

Fig. 5 Diagram showing the wilting symptoms and wilting indices of control strain CMM101 compared with the *chpC* mutant (CMM101*chpC*β), the *chpG* mutant (CMM101*chpG*β) and the complemented *chpC* mutant (CMM101*chpC*β–pIG216Cβ). Plants with beginning leaf curling just at the tips are indicated by a '(+)', obviously curled leaves by a '+' and strong wilting symptoms were recorded as '++' (when at least two-thirds of the leaves showed wilting symptoms). When plants showed severe wilting symptoms they were termed 'dead'. The wilting index (WI) is indicated and defined as the number of days required for 50% of the plants to show clear wilting symptoms '+'.

Table 1 *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) strains and their plasmid status, wilting indices and titre.

Strain	Plasmid(s)	Wilting index (d)*	Titre (cfu/g plant homogenate)†	SD	Reduction of titre‡
Cmm NCPPB 382	pCM1, pCM2	12	7.4×10^9§	$\pm 7.3 \times 10^9$	–
CMM101	pCM1	15–16	9.9×10^9§	$\pm 4.1 \times 10^9$	–
CMM101chpCβ	pCM1	–	2.0×10^7¶	$\pm 3.2 \times 10^7$	495
CMM101chpCβ pIG216Cβ	pCM1 pIG216Cβ	17	1.9×10^9¶	$\pm 1.7 \times 10^9$	5.2
CMM101chpGβ	pCM1	15–16	1.8×10^9§	$\pm 2.4 \times 10^9$	5.5

*Time post-infection when 50% of the infected plants show wilting symptoms, i.e. curled leaves; $n = 64$ plants.
†After 28 days.
‡*x*-fold reduction of bacterial titre compared with CMM101.
§$n = 10$ plants.
¶$n = 20$ plants.

protease encoded by *chpG* is also involved in plant–microbe interaction.

Complementation of the *chpC* mutant with plasmid pIG216Cβ

To confirm that the phenotype of the CMM101 *chpC*β mutant was not caused by some other mutation in the chromosome, a merozygote containing the inactivated and the wild-type *chpC* gene was constructed. A 2.5-kb *Eco*RV fragment containing the complete *chpC* of cmis2p456d03 was cloned into the *Eco*RV-digested *E. coli–Cmm* shuttle vector pHN216 (Laine *et al.*, 1996), resulting in plasmid pIG216Cβ, which was introduced into the *chpC* mutant by electroporation. Plasmid DNA from neomycin-resistant colonies was isolated and the presence of the plasmids was verified by *Nco*I digestion (data not shown). One clone carrying the hybrid plasmid pIG216Cβ designated CMM101 *chpC*β-pIG216Cβ was chosen for assays on tomato plants.

In the virulence assay 64 tomato plants were infected with the merozygotic strain CMM101 *chpC*β-pIG216Cβ and scored daily for disease symptoms. The virulence of strain CMM101 *chpC*β-pIG216Cβ was almost identical to the control strain CMM101 with a wilting index of 17 days (Fig. 5). In addition, the titre *in planta* of the complemented *chpC* mutant was close to that of

CMM101 (Table 1). This indicates that trans-complementation of the *chpC* mutation by the wild-type gene had occurred and no other mutation or rearrangement in the chromosome of *Cmm* was responsible for the phenotype of the CMM101 *chpC*β mutant.

DISCUSSION

One of the virulence factors of *Cmm* NCPPB382 is the putative serine protease Pat-1, which is encoded by plasmid pCM2 (Dreier *et al.*, 1997). The exact function of this serine protease and its substrate remains unknown. The first identified Pat-1 homologues, *phpA*, *phpB* on plasmid pCM2 and *chpA* on the chromosome, were found by hybridizations against a *pat-1* probe (Burger *et al.*, 2005). Six further proteins homologous to Pat-1 (ChpB–ChpG) were found to be encoded in a 50-kb region of the *Cmm* chromosome.

Most microbial proteases are secreted enzymes and can be classified based on the essential catalytic residue at their active site. They include aspartate proteases, cysteine proteases, metalloproteases and serine proteases. Pat-1 and the homologous proteins may comprise a family of serine proteases belonging to the chymotrypsin subfamily S1A (Fig. 2). At the N-terminus they all have a signal peptide indicating that these proteins may be secreted. Some of them also share the hypothetical processing site AQA↓V (Burger *et al.*, 2005). Furthermore, several cysteine residues are conserved as is also the case in some other members of the serine protease subfamily S1A. It is possible that cysteines form disulphide bridges in the mature enzymes. As the *chpA*, *chpB* and *chpD* genes contain frame shifts or in-frame stop codons, only truncated and possibly non-functional proteins originate from these genes. The other genes *chpC*, *chpE*, *chpF* and *chpG* may encode functional serine proteases; however, when *Cmm* is grown in rich or minimal media the supernatant contains no protease activity when tested with common serine protease substrates, such as casein, azocasein and azocoll (Burger *et al.*, 2005). This indicates that these putative serine proteases might have a specific activity or are only expressed in the plant. Holtsmark *et al.* (2008) recently demonstrated that *pat-1* homologous genes in *Clavibacter michiganensis* subsp. *sepedonicus* were also expressed in rich medium. However, depending on which *pat-1* homologous gene was tested the transcript level decreased or increased during infection of the host plant, indicating that expression of these genes is indeed affected in the interaction with the host tomato.

To elucidate the possible function of these serine proteases we examined whether they are involved in pathogenicity, as is the case for Pat-1. Thus far, we have only succeeded to inactivate the genes *chpC* and *chpG* by insertion of an antibiotic resistance cassette and exchange of the wild-type gene by the inactivated one. Inactivation of the *chpG* gene had no effect on virulence or colonization, while knock-out of the *chpC* gene resulted in a significant reduction in virulence and bacterial growth *in planta*. The titre of CMM101 *chpC*β *in planta* 28 dpi was only 2×10^7 cfu/g plant homogenate as compared with 9.9×10^9 cfu/g plant homogenate for CMM101 and only seven of 64 plants showed curled leaves, the mildest manifestation of disease. Thus, a correlation seems to exist between development of wilting symptoms and the bacterial titre *in planta*. Assays with several *Cmm* strains and mutants showed that only when titres of higher than 10^8 bacteria/g plant homogenate are reached is wilting of tomato plants observed (data not shown). *Cmm* grows in the xylem fluid and a slow bacterial growth *in planta* may be caused by a lack of nutrients, especially as *Cmm* is auxotrophic for methionine, thiamine and nicotinic acid. Thus, these compounds and sugars or organic acids have to be provided in the xylem in sufficient concentration to allow growth of *Cmm*. It is possible that the ChpC serine protease degrades or processes specific proteins of either plant or bacterial origin to enrich the xylem fluid with nutrients (perhaps methionine) or that ChpC generates a signal which induces the plant to secrete nutrients into the xylem fluid. In order to test this hypothesis, it will be necessary to study the composition of the xylem fluid in infected and non-infected tomato plants.

Many plant pathogens producing proteases have been studied and the results suggest that proteases seem to play a role in virulence. However, often neither the exact mode of action nor the substrate or target of the proteases is known. An example is the interference with regulatory mechanisms of the plant by cysteine proteases of proteobacteria translocated into the plant cell via the type III secretion system. The proteases XopD, AvrXv4, AvrPphB and AvrRpt2 were shown to hydrolyse specific host target proteins (Hotson and Mudgett, 2004; Hotson *et al.*, 2003). XopD of *Xanthomonas campestris* pv. *vesicatoria* disturbs plant signal transduction as it mimics plant-specific SUMO (small ubiquitin-like modifier) proteases responsible for the activation or degradation of proteins destined to be SUMOylated. *In planta* analysis showed that XopD targets numerous SUMO-modified proteins and therefore may affect several plant signalling pathways leading to stress responses or defence reactions against pathogens (Hanania *et al.*, 1999; Hotson *et al.*, 2003). However, thus far we have no indication for a type III secretion system in *Cmm*. In spite of this, ChpC may still be a similar effector which is transported into the plant cell by a different transport system.

Filamentous fungi secrete a broad range of proteolytic enzymes during penetration and colonization of the plant tissue. The infection of potato tubers with *Fusarium eumartii* is accompanied by an accumulation of a serine protease activity (Olivieri *et al.*, 1998), which suggests that this protease activity might be involved in plant–pathogen interaction (Olivieri *et al.*, 2002). In fact, the extracellular serine protease of *Fusarium eumartii* belonging to the subtilase subfamily was shown to degrade potato pathogenesis-related (PR) proteins as well as specific polypeptides in the intercellular washing fluid and cell-wall proteins from potato tubers

(Olivieri *et al.*, 2002). It is possible that ChpC serine protease plays a similar role in the interaction with tomato plants.

Altogether, the importance of proteases as virulence factors remains unclear because in many cases the inactivation of proteases by deletion, mutation or disruption did not affect pathogenicity (Jaton-Ogay *et al.*, 1994). This seems to be the case for the putative serine protease ChpG, but for ChpC we can clearly show that it has an effect on the interaction of *Cmm* with tomato plants. One of the main goals for the future will be the identification of substrate molecules for the putative Chp serine proteases in order to obtain clues to their function in the *Clavibacter*–tomato interaction.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and media

Bacterial strains and plasmids used and constructed in this study are listed in Table 1. Strains of *Cmm* were grown at 25 °C on C-medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 5 g/L glucose, pH 7.2) or M9 medium (6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 1 g/L NH_4Cl, 0.5 g/L NaCl, 1 mM $MgSO_4$, 0.01 mM $CaCl_2$, 200 mg/L methionine, 200 mg/L thiamine, 20 mg/L nicotinic acid, 2 g/L glucose). *E. coli* was grown on TBY medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.2) at 37 °C. When selecting for antibiotic resistance to *E. coli*, media contained 10 μg/mL chloramphenicol or 50 μg/mL kanamycin. After electroporation strains of *Cmm* were grown on SB medium (10 g/L tryptone, 5 g/L yeast extract, 4 g/L NaCl, 0.5 M sorbitol, 20 mM $MgCl_2$, 20 mM $CaCl_2$). Selection for chloramphenicol and/or neomycin resistance was achieved with SB medium supplemented with these antibiotics at 10 and 75 μg/mL, respectively.

DNA isolation, manipulation and transfer

Plasmid DNA of *E. coli* used for cloning and electroporation was isolated and purified with QIAprep spin columns as specified by the manufacturer (QIAGEN, Hilden, Germany). Preparation of total DNA for hybridization was done as described by Hopwood *et al.* (1985). Plasmid DNA of *Cmm* was isolated by the method of Birnboim and Doly (1979) with the following modifications. Ten-millilitre cultures were harvested, washed with 2 mL 10% glycerol, and frozen at –20 °C overnight. The pellet was resuspended in 300 μL P1 buffer (50 mM Tris-HCl, 10 mM EDTA, 100 μg/mL RNaseA, pH 8) with addition of 7 mg/mL lysozyme. Lysis was performed with 300 μL buffer P2 (200 mM NaOH, 1% SDS) for 5 min. Three hundred microlitres of buffer P3 (3 M KAc, pH 5.5) was added to neutralize the suspension. DNA hydrolysis with restriction endonucleases, ligation and transformation were carried out by standard procedures (Sambrook *et al.*, 1989). Isolation of restriction fragments from agarose gels was done with the QIAquick Gel Extraction Kit as specified by the manufacturer (QIAGEN).

Sequence analysis

Amino acid sequence alignment was performed with the alignment programs ClustalX (Thompson *et al.*, 1997) and GeneDoc (Nicholas *et al.*, 1997). Signal sequences were predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP, Nielson *et al.*, 1997).

Construction of plasmids for gene replacement and complementation

The plasmid cmis2p0456d03 carrying the native complete *chp*C gene was used for the construction of a mutagenesis vector. Cmis2p0456d03 cannot replicate in *Cmm*. Plasmid pIGCβ was constructed by cloning the chloramphenicol resistance gene *cmx* of pEC70 (Tauch *et al.*, 1998) as a 1.9-kb *Bsa*AI fragment into the unique *Msc*I restriction site of cmis2p0456d03 within the *chpC* gene.

In plasmid cmis2p0456h08, which carries the native *chpG* gene, a 640-bp *Eco*47III fragment containing the promoter region and part of *chpG* was replaced by the 1.9-kb *Bsa*AI fragment of pEC70 carrying the *cmx* gene (pIGGβ) (Fig. 1).

Plasmid pIG216Cβ used for complementation was constructed based on the *E. coli–Cmm* shuttle vector pHN216 (Laine *et al.*, 1996). The 2.5-kb *Eco*RV fragment of cmis2p0456d03 carrying the *chpC* gene was integrated into the vector pHN216 linearized by *Eco*RV.

Electroporation

Electroporation into *E. coli* was conducted according to the Gene Pulser manual (Bio-Rad Laboratories, Krefeld, Germany). The electroporation into *Cmm* was performed as described by Kirchner *et al.* (2001).

Southern blot analysis

An internal 465-bp fragment of *chpC* was generated by PCR (primer: chpC-1: CCCCATCGGAACGGTTTATTGG and chpC-2: GCTCTGCTCTGTGAGACGATG). Amplification of the 465-bp fragment was done according to the following conditions: 4 min at 94 °C, 35 cycles of 1.5 min at 94 °C, 1.5 min at 64 °C and 1.5 min at 72 °C, and finally 10 min at 72 °C. DNA was labelled using the random primed DNA labelling kit (Roche Diagnostics, Mannheim, Germany). For *chpG* the 2.3-kb *Eco*RV fragment of cmis2p0456h08 was used as a probe. The digoxygenin-11-dUTP-labelled 1.9-kb *Bsa*AI fragment of pEC70 was used to detect the *cmx* gene. pUC18 DNA was labelled with digoxigenin-11-dUTP by nick translation (Sambrook *et al.*, 1989). Digested chromosomal and

plasmid DNA fragments were separated on 0.8–1.0% agarose gels and transferred to nylon membranes (porablot, Macherey & Nagel, Düren, Germany; or Nytrans, Schleicher & Schuell, Dassel, Germany) by capillary blots (Smith and Summers, 1980). Hybridizations were carried out at 68 °C overnight in a buffer containing 5× SSC (0.75 M NaCl plus 0.075 M sodium citrate), 0.02% sodium dodecyl sulphate, 0.1% Na lauroylsarcosyl, and 2% blocking reagent (Roche Diagnostics). The nylon membrane was washed twice with 0.1× SSC, 0.1% sodium dodecyl sulphate at 68 °C for 15 min. Detection was carried out as recommended by the manufacturer with anti-DIG-AP conjugate and NBT and BCIP as chromogenic substrates (Roche Diagnostics).

Virulence assay

Tomato plants (*Lycopersicon esculentum* cv. Moneymaker) used for the standard infection procedure were about 14 days old (two-leaf stage). Fresh *Cmm* cultures, which were grown at 28 °C on rich medium, were harvested by centrifugation and adjusted to an optical density (580 nm) of 8–9 with sterile water. After removal of adhering soil from the roots, the plants were immersed for 15–20 min in the high-titre bacterial suspension to achieve an effective infection, which under these conditions is about 70–90%. The infected plants were then planted into sterile soil. Growth of the plants took place at 25 °C with a day–night rhythm of 16 h day at 120 000 lx and 8 h night, and 40–50% relative air humidity. Each experimental group consisted of 64 plants. Disease symptoms were estimated by scoring infected plants for wilting symptoms. The wilting index is defined as the number of days required for 50% of the plants to display wilting symptoms (leaf curling) (Meletzus *et al.*, 1993). Parallel to this, plants were examined daily over a period of up to 28 days for the development of wilting symptoms. Plants with beginning leaf curling just at the tips were assigned the score '(+)', obviously curled leaves were '+' and strong wilting symptoms were recorded as '++' (when at least two-thirds of the leaves showed wilting symptoms). When all leaves showed wilting and photosynthesis was no longer possible, the plants were termed 'dead'.

Determination of bacterial titres in planta

Twenty-eight days after infection 10–20 tomato plants were harvested by cutting the stem 1 cm above the soil. Single plants were frozen in liquid nitrogen, ground to powder in a sterile mortar and then suspended in buffer (7 g/L $Na_2HPO_4 \cdot 2H_2O$, 5 g/L NaCl, 3 g/L KH_2PO_4, pH 7) (1 mL buffer/g fresh weight). Appropriate dilutions were plated on selective medium and incubated at 25 °C for 3–5 days to determine the number of colony forming units (cfu). The mean of the 10–20 individual titre determinations was taken to calculate the *in planta* titre and the standard deviation.

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