Requirement of a *mip*-Like Gene for Virulence in the Phytopathogenic Bacterium *Xanthomonas campestris* pv. *campestris*

Ning Zang,^{1,2} Dong-Jie Tang,² Mei-Liang Wei,² Yong-Qiang He,² Baoshan Chen,² Jia-Xun Feng,² Jing Xu,² Yong-Qi Gan,² Bo-Le Jiang,² and Ji-Liang Tang²

¹College of Agriculture and Biotechnology, Zhejiang University, Hangzhou 310029, China; ²Guangxi Key Laboratory of Subtropical Bioresources Conservation and Utilization, The Key Laboratory of Ministry of Education for Microbial and Plant Genetic Engineering, and College of Life Science and Technology, Guangxi University, 100 Daxue Road, Nanning Guangxi 530004, China

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Macrophage infectivity potentiators (Mips) are FKBP domain-containing proteins reported as virulence factors in several human pathogens, such as members of genera *Legionella*, *Salmonella* and *Chlamydia*. The putative peptidyl-prolyl *cis-trans* isomerase (PPIase) encoded by XC2699 of the plant bacterial pathogen *Xanthomonas campestris* pv. *campestris* 8004 exhibits a 49% similarity at the amino-acid level to the Mip protein of *Legionella pneumophila*. This *mip*-like gene, XC2699, was overexpressed in *Escherichia coli* and the purified (His)₆-tagged Mip-like protein encoded by XC2699 exhibited a PPIase activity specifically inhibited by FK-506. A mutation in the *mip*-like gene XC2699 led to significant reductions in virulence and replication capacity in the host plant Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.). Furthermore, the production of exopolysaccharide and the activity of extracellular proteases, virulence factors of *X. campestris* pv. *campestris*, were significantly decreased in the *mip*-like mutant. These results reveal that the *mip*-like gene is involved in the pathogenesis of *X. campestris* pv. *campestris* through an effect on the production of these virulence factors.

A Mip (macrophage infectivity potentiator) was originally identified as an important virulence factor for *Legionella pneumophila*, the causative agent of Legionnaires' disease (Cianciotto et al. 1989). The *L. pneumophila* Mip is a 24-kDa cell-surface protein and exhibits a PPIase activity that is specifically inhibited by FK-506 (Cianciotto et al. 1989; Fischer et al. 1992). Mip is highly conserved (82 to 99% identity) in the *Legionella* genus (Ratcliff et al. 1997), and Mip-like proteins or *mip*-related DNA sequences have also been found in other pathogenic organisms, including the human pathogenic organisms *Chlamydia trachomatis* (Lundemose et al. 1993), *Chlamydia psittaci* (Rockey et al. 1996), *Coxiella burnetii* (Mo et al. 1995), *Trypanosoma cruzi* (Moro et al. 1995), *Salmonella typhimurium* (Horne et al. 1997), and *Neisseria gonorrhoeae* (Leuzzi et al. 2005), as well as the plant-pathogenic bacterium *Xylella fastidiosa* (Van Sluys et al. 2003). Although originally identified as a virulence factor in many pathogens, Mip is not

unique to pathogenic organisms; *mip*-like genes also exist in nonpathogenic organisms such as *Escherichia coli* and other members of family *Enterobacteriaceae* (Horne and Young 1995; Rahfeld et al. 1996). In particular, two Mip-like proteins, FkpA and FKBP22 (also known as FklB), are found in *E. coli*, and unlike *L. pneumophila* Mip, which is a cell-surface protein, FkpA and FKBP22 are located in the periplasm of the *E. coli* cell (Horne and Young 1995; Rahfeld et al. 1996).

The wide distribution and different cellular localization of Mip-like proteins may reflect the significant and different cellular functions of this protein. In *L. pneumophila*, mutation in the *mip* gene led to defective ability to infect both human phagocytes and freshwater protozoa (Cianciotto et al. 1989). In addition to *L. pneumophila*, an involvement of Mip or Mip-like proteins in microbial pathogenesis has also been reported in *L. micdadei* (O'Connell et al. 1996), *L. longbeachae* (Doyle et al. 1998), *Chlamydia trachomatis* (Lundemose et al. 1993), *T. cruzi* (Moro et al. 1995), *S. typhimurium* (Horne et al. 1997), and *N. gonorrhoeae* (Leuzzi et al. 2005). In addition to their role in pathogenesis, Mip-like proteins also play roles in other cellular processes. It has been proposed that FkpA, a Mip-like protein in the nonpathogenic bacterium *E. coli*, is a heat-shock protein that functions as a chaperone for envelope proteins in the periplasm and is involved in the stress response (Arie et al. 2001).

A Mip or Mip-like protein consists of two parts: an N-terminal region (100 to 150 amino-acid residues), which is responsible for the dimerization of the protein, and a C-terminal region (approximately 100 amino-acid residues), a human FK506-binding protein FKBP12-homologous domain that is responsible for the PPIase activity (Riboldi-Tunnichiffe et al. 2001). Like the Mip of *L. pneumophila*, the Mip-like proteins from *Chlamydia trachomatis*, *Coxiella burnetii*, *E. coli*, *T. cruzi*, and *N. gonorrhoeae* also exhibit PPIase activity inhibitable by FK506 and rapamycin (Leuzzi et al. 2005; Lundemose et al. 1993; Mo et al. 1995; Moro et al. 1995; Rahfeld et al. 1996). Although PPIase activity is associated with the acceleration of protein folding, the mechanism by which Mip is involved in microbial pathogenesis is still unknown, and the relevance of the PPIase activity to the virulence function of Mip is not well understood. Wintermeyer and associates (1995) reported that the PPIase activity of the Mip protein does not play a role in *Legionella* virulence. Recently however, Helbig and associates (2003) demonstrated that an intact PPIase active site is essential for the virulence-enhancing property of *Legionella* Mip

Ning Zang and Dong-Jie Tang contributed equally to this work.

Corresponding author: J.-L. Tang;
E-mail: jlt@gx163.net or jltang@gxu.edu.cn

protein and Kohler and associates (2003) found that the N-terminal and dimerization of Mip are necessary for full virulence in both *Acanthamoeba castellanii* and guinea pigs but that the PPIase activity is only necessary for full virulence in guinea pigs and not in *Acanthamoeba castellanii*. In *Chlamydia trachomatis*, the PPIase activity of the Mip-like protein is required for successful infection (Lundemose et al. 1993).

Although *mip*-related DNA sequences are widely distributed among prokaryotes and the Mip protein seems to be a common virulence factor for many human pathogens, not much about either Mip or Mip-like proteins in plant pathogens is presently known. To our best knowledge, the only *mip* or *mip*-like gene reported for a plant parasite to date is the one annotated in the genome of *Xylella fastidiosa* (Van Sluys et al. 2003). In this paper, we report the identification and characterization of a *mip*-like gene of *Xanthomonas campestris* pv. *campestris*, the causal agent of the black rot disease of cruciferous plants (Williams 1980) and demonstrate that this gene is required for virulence and the production of exopolysaccharide as well as extracellular protease activity.

RESULTS

The open reading frame (ORF) XC2699 of *X. campestris* pv. *campestris* 8004 encodes a Mip-like protein.

A *mip* gene has been annotated in *Xylella fastidiosa* (Van Sluys et al. 2003), a plant bacterial pathogen that belongs to the *Xanthomonas* group. Thus, it is possible that a *mip*-like gene may also exist in *X. campestris* pv. *campestris*, also belonging to the same group. Since no gene was annotated as either *mip* or *mip*-like in *X. campestris* pv. *campestris* ATCC33913 (da Silva et al. 2002) or strain 8004 (Qian et al. 2005), we performed a BLAST search (Altschul et al. 1990) against the *X. campestris* pv. *campestris* 8004 genome with the *Xylella fastidiosa* *mip* gene, finding that the ORF XC2699 (Fig. 1) (GenBank accession number NC_004556), annotated as a PPIase (Qian et al. 2005), was the homolog of the *mip* of *Xylella fastidiosa*, sharing 72% identity at the nucleotide level. The deduced amino acids of the ORF XC2699 of *X. campestris* pv. *campestris* 8004 showed 85 and 49% amino-acid similarity to the putative Mip protein of *Xylella fastidiosa* and the Mip protein of *L. pneumophila*, respectively (Fig. 2).

X. campestris pv. *campestris* Mip-like protein is an FKBP-type PPIase.

Based on the sensitivity to the immunosuppressive drugs FK506, rapamycin, or cyclosporin A, PPIases are classified into three types: the FK506 binding proteins (FKBP), cyclophilins, and parvulins (Shaw 2002). These three types of PPIase share no sequence similarity. FKBP bind specifically to and are inhibited by FK506 and rapamycin, whereas cyclophilins bind to and are inhibited by cyclosporin A. For parvulins, no

inhibitor has yet been identified. It has been reported that Mip or Mip-like proteins from *L. pneumophila*, *Chlamydia trachomatis*, *Coxiella burnetii*, *E. coli*, and *T. cruzi* are FKBP-type PPIases (Fischer et al. 1992; Lundemose et al. 1993; Mo et al. 1995; Moro et al. 1995, Rahfeld et al. 1996). To determine if *X. campestris* pv. *campestris* Mip-like protein has PPIase activity, the *mip*-like gene XC2699 was overexpressed in *E. coli* BL21 (DE3)pLysS, and fusion protein with a (His)₆ tag was purified using a Ni-NTA column (Fig. 3). The PPIase activity of the purified protein was determined at 10°C by protease-coupled assay as described (Fischer et al. 1989), using N-Suc-AlaAlaProPhe p-nitroanilide as the substrate. The time course of the enzymatic reaction clearly showed the accumulation of 4-nitroanilide, the product of chymotryptic cleavage, when the purified protein was added to the reaction mixture (Fig 4), demonstrating the PPIase activity of the protein.

The effect of the immunosuppressants FK506 and cyclosporin A on the PPIase activity of the enzyme was tested. As shown in Figure 5, the relative PPIase activity of the (His)₆ Mip-like protein decreased to 38% when FK506 was added at 5 µM in the reaction mixture and dropped to near zero at 15 µM of FK506. In contrast, the PPIase activity of the protein was not affected by cyclosporin A, even at a concentration of 25 µM. These results demonstrate that *X. campestris* pv. *campestris* Mip-like protein is an FKBP-type PPIase.

L. pneumophila Mip has been demonstrated to be a cell surface protein (Cianciotto et al. 1989; Fischer et al. 1992), and the two Mip-like proteins FkpA and FKBP22 in *E. coli* are located in the periplasm (Horne et al. 1995; Rahfeld et al. 1996). The result of signal peptide prediction using SignalP 3.0 software (Bendtsen et al. 2004) showed that the *X. campestris* pv. *campestris* Mip-like protein contains a signal peptide sequence at its N-terminal (data not shown). Taking all this information together, it is possible that the *X. campestris* pv. *campestris* Mip-like protein is a periplasmic or secreted protein. To test this possibility, the PPIase activity in the culture supernatant and the periplasmic fraction of the *X. campestris* pv. *campestris* wild-type strain 8004 and the *mip*-like mutant NK2699 was tested using the method described above. The result revealed the PPIase activity in the culture supernatant of both strains 8004 and NK2699 to be undetectable, whereas the PPIase activity in the periplasmic fraction of the wild-type strain 8004 is approximately fourfold higher than that of the *mip*-like mutant NK2699 (Table 1). This result suggests that the *X. campestris* pv. *campestris* Mip-like protein is a periplasmic FKBP-type PPIase.

The *mip*-like gene XC2699 is required for the full virulence of *X. campestris* pv. *campestris*.

To investigate the biological function of the *mip*-like gene in *X. campestris* pv. *campestris*, XC2699 was mutated by homologous suicide plasmid integration. A total of 35 kanamycin- and rifampicin-resistant transconjugants were obtained in a tripar-

Fig. 1. Schematic presentation of the genetic organization of the XC2699 locus and the DNA fragment used for XC2699 mutant complementation. The far left of the fragment used for complementation was beyond the stop codon of XC2699 by 600 bp (reaching into the coding region of XC2698 by 509 bp), and the far right beyond the start codon of XC2699 by 300 bp (reaching into the coding region of XC2700 by 135 bp). XC2698 and XC2700 were annotated as 'MutT/nudix family protein' and '3-hydroxybutyryl-CoA dehydratase,' respectively.

ental mating experiment, and six transconjugants were randomly selected for confirmation by polymerase chain reaction (PCR) and sequencing. All of them were confirmed to have a plasmid integrated in XC2699 (data not shown). A representative mutant was chosen and named NK2699 (Table 2). The virulence of the mutant NK2699 was tested on the host plant Chinese radish (*Raphanus sativus* L. var. *radiculus* Pers.) by the leaf-clipping method (Dow et al. 2003). Ten days postinoculation, a lesion length with a mean of 2.3 mm was observed on the leaves inoculated with the mutant NK2699, while a lesion length with a mean of 15.1 mm was observed on leaves inoculated with the wild-type strain 8004, and the attenuated virulence of the mutant could be restored by the wild-type *mip*-like gene XC2699 in the *trans* configuration (Fig. 6). As analyzed by *t*-test, the mean lesion length caused by the *mip*-like mutant NK2699 was significantly shorter than that caused by the wild-type strain at $P = 0.01$ (*t*-test). Meanwhile, the lesion lengths of the complemented strain CNK2699 and the wild-type strain were not significantly different at $P = 0.05$. These results revealed that the *mip*-like gene is indeed required for the full virulence of *X. campestris* pv. *campestris*.

It has been reported that mutation in the *mip* (or *mip*-like) gene of *L. pneumophila*, *N. gonorrhoeae*, or *S. typhimurium*

results in a decrease in the proliferation of these pathogens inside the infected tissues (Cianciotto et al. 1989; Horne et al. 1997; Leuzzi et al. 2005). To test whether mutation in the *X. campestris* pv. *campestris* *mip*-like gene has a similar effect, the mutant bacteria were recovered from the inoculated leaves (using the same inoculation method and cell concentration as that of the virulence assay). Although the growth rate of the *mip*-like mutant NK2699 is similar to that of the wild-type strain 8004 in NYG medium (5 g of peptone, 3 g of yeast extract, and 20 g of glycerol per liter) (data not shown), the number of the mutant bacterial cells recovered from the infected leaves was significantly lower than that of the wild-type strain at each of the test points ($P = 0.01$) and was approximately 100-fold less than that of the wild-type strain two days postinoculation and thereafter (Fig. 6). The growth capacity of the mutant in planta could be completely restored by the wild-type *mip*-like gene XC2699 in *trans* (Fig. 6). These results demonstrate that the *mip*-like gene of *X. campestris* pv. *campestris* is important for the competent growth of the pathogen in planta.

To define whether mutation in the *mip*-like gene of *X. campestris* pv. *campestris* has any effect on the hypersensitive response (HR) of *X. campestris* pv. *campestris*, the *mip*-like

Fig. 2. Alignment of the amino-acid sequences of Mip homologs from *Xanthomonas campestris* pv. *campestris* (XC2699), *Xylella fastidiosa* Temecula1 (XfMip), and *Legionella pneumophila* (LpMip). The sequences of these proteins were retrieved from GenBank with the following accession numbers: YP_243768 for XC2699, NP_779719 for *Xylella fastidiosa* Temecula1 Mip, and YP_126188 for *L. pneumophila* Mip). Black boxes denote identical amino-acid residues, whereas gray-shaded boxes denote similar residues.

mutant NK2699 was inoculated on the nonhost plant pepper ECW-10R. NK2699 caused a wild-type HR (data not shown), suggesting that the *mip*-like gene of *X. campestris* pv. *campestris* is not required for HR.

The *mip*-like gene of *X. campestris* pv. *campestris* is required for the production of exopolysaccharide and the activity of extracellular protease.

Exopolysaccharide is one of the major virulence determinants in *X. campestris* pv. *campestris*, and extracellular protease, amylase, and endoglucanase enzymes have been implicated in

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the purified recombinant Mip-like protein of *Xanthomonas campestris* pv. *campestris*. Lanes 1 and 2, crude extracts of *Escherichia coli* BL21(DE3)pLysS/pET-30-2699 before (lane 1) and after (lane 2) induction with IPTG; lane 3, the purified (His)₆-Mip-like protein; lane M, molecular mass markers.

Fig. 4. The timecourse of the enzymatic reaction with the purified (His)₆-Mip-like protein. Reaction was performed in 1 ml of 35 mM Hepes buffer (pH 7.8) at 10°C with the addition of Succinyl-Ala-Ala-Pro-Phe-pnitroanilide as substrate at a final concentration of 0.05 mM and 19 µg of (His)₆-Mip-like protein as the enzyme. The reaction product of chymotryptic cleavage, 4-nitroanilide, was detected by measuring the absorbance at 390 nm at intervals of 60 s. In the control reaction, the purified (His)₆-Mip-like protein was substituted with bovine serum albumin (BSA) protein. The data presented are from a representative experiment, and similar results were obtained in two additional independent experiments. ■ represents the Mip-like protein and ● the BSA protein.

contributing to *X. campestris* pv. *campestris* virulence (Chan and Goodwin 1999). For further investigation of the reason why mutation in *mip*-like genes would lead to a reduction in virulence of *X. campestris* pv. *campestris*, the production of these virulence factors in a *mip*-like gene mutant was tested. Measurement of the production of exopolysaccharide and extracellular enzymes revealed that a mutation in the *mip*-like gene led to a significant reduction in the production of exopolysaccharide and almost abolished the extracellular protease activity, while the extracellular endoglucanase and amylase activity is only slightly affected by *mip*-like gene mutation (Table 3; Fig. 7). These defective phenotypes of the mutant were restored by the *mip*-like gene XC2699 in *trans* (Table 3; Fig. 7). This result demonstrated that the *mip*-like gene is required for the production of exopolysaccharide and the activity of extracellular protease in *X. campestris* pv. *campestris*.

DISCUSSION

Mip and Mip-like proteins have been demonstrated to be an important virulence factor in several human pathogens (Cianciotto et al. 1989; Doyle et al. 1998; Leuzz et al. 2005; Lundemose et al. 1993; Moro et al. 1995; O'Connell et al. 1996). In this report, we demonstrate that the Mip-like protein also plays an important role in the pathogenesis of the plant pathogenic bacteria *X. campestris* pv. *campestris*.

Mip protein was first described as an important virulence factor of the obligate intracellular human pathogen *L. pneumophila* (Cianciotto et al. 1989). In *L. pneumophila*, inactivation of the *mip* gene leads to a significant reduction in virulence of

Fig. 5. Effect of FK-506 (▲) and cyclosporin A (●) on the PPIase activity of the purified (His)₆-Mip-like protein. The data presented are from a representative experiment, and similar results were obtained in two other independent experiments.

Table 1. PPIase activity in the culture supernatant and the periplasmic fraction of *Xanthomonas campestris* pv. *campestris*

Strains	PPIase activities (A ₃₉₀) ^a	
	Culture supernatant	Periplasmic fraction
8004	NC	0.788 ± 0.06a
NK2699	NC	0.190 ± 0.08b

^a A 100-µl aliquot of the culture supernatant (4 mg total protein/ml) or periplasmic fraction (4 mg total protein/ml) were added to 1 ml of reaction mixture, and the absorbance at 390 nm (A₃₉₀) was measured after incubation at 10°C for 10 min (at 0 min, the A₃₉₀ = 0). Data are the mean ± standard deviation of triplicate measurements; the different letters in each data column indicate significant differences at *P* = 0.01. The data presented were from a representative experiment and similar results were obtained in two other independent experiments. NC = no change in A₃₉₀.

the pathogen in an animal model (Cianciotto et al. 1990), and further investigation found that *L. pneumophila* Mip is necessary for proliferation of this pathogen inside infected tissues such as human alveolar macrophages, macrophage-like cell line U937, and protozoan cells (Cianciotto and Fields 1992; Cianciotto et al. 1989, 1995). As with *L. pneumophila*, a mutation in the *mip*-like gene *fkpA* of *S. typhimurium* Copenhagen and the *mip* gene of *N. gonorrhoeae* also reduces survival of these pathogens within macrophages (Horne et al. 1997; Leuzzi et al. 2005). These results suggest that Mips or Mip-like proteins in diverse pathogens play an important role in proliferation inside their hosts. However, the exact mechanism by which Mip and Mip-like protein contributes to pathogen proliferation inside infected tissue is still a subject for further investigation.

In this study, we found that inactivation of a *mip*-like gene in the plant bacterial pathogen *X. campestris* pv. *campestris* led to a significant reduction in virulence in the host plant Chinese radish and dramatically decreased bacterial replication inside the host. Interestingly, unlike *L. pneumophila*, in which *mip* mutations confer reduced infectivity in the host but no other phenotypes are observed, in *X. campestris* pv. *campestris*, the production of exopolysaccharide and the activity of extracellular protease were significantly decreased in the *mip*-like mutant. Because exopolysaccharide and extracellular protease have been demonstrated to be virulence factors of *X. campestris* pv. *campestris* and required for the competent growth of this pathogen inside the host (Chan and Goodwin 1999), the reduced virulence and impaired in planta multiplication of the *mip*-like mutant of *X. campestris* pv. *campestris* can be, at least partially, explained by the reduction in the production of exopolysaccharide and the lost of activity of extracellular protease. A periplasmic PPIase, RotA, which belongs to the cyclophilin-type PPIase family, has been characterized in another plant bacterial pathogen, *Erwinia chrysanthemi*; but unlike the *X. campestris* pv. *campestris* Mip-like protein, inactivation of *E. chrysanthemi* *rotA* had no effect on the production of virulence factors, such as pectic lyase, of this pathogen (Pissavin and Hugouvieux-Cotte-Pattat 1997).

The mechanism by which the Mip-like protein of *X. campestris* pv. *campestris* affects the production of exopolysaccharide and the activity of extracellular protease is unclear, but there exist several possibilities. i) The expression of genes involved in the production of exopolysaccharide and extracellular protease in *X. campestris* pv. *campestris* is affected by *mip*-like gene mutation. ii) It acts as a foldase so is required for the correct folding of factors necessary for the secretion of exopolysaccharide and extracellular protease. iii) In *X. cam-*

pestris pv. *campestris*, the Mip-like protein is required for the correct folding of extracellular protease in the periplasm. iv) Either one or both the specific activity or substrate sensitivity of the extracellular protease is affected by *mip*-like gene mutation. Determining the actual mechanism needs further investigation.

The cellular location of Mip and Mip-like protein may be related to its function. *L. pneumophila* Mip is a cell surface protein (Cianciotto et al. 1989) that accumulates in multilamellar host membranes (Helbig et al. 2001), *N. gonorrhoeae* Mip is also a cell surface protein (Leuzzi et al. 2005), and the Mip-like protein of *T. cruzi* was found to be secreted into the cell culture medium in the infective stage (Moro et al. 1995). Thus, as a surface or secreted protein, Mip-like proteins may directly interact with their host. Although the function of Mip and Mip-like protein during infection is still unknown, it has been proposed that *L. pneumophila* Mip protein may contribute to the competent proliferation of the pathogen inside macrophages either through prevention of phagosome-lysosome fusion (Cianciotto et al. 1989) or inhibition of host defense systems (Hacker and Fischer 1993). Although our data suggest that *X. campestris* pv. *campestris* Mip-like protein is located in the periplasm, Watt and associates (2005) reported recently that, in *X. campestris* pv. *campestris* B100, a putative PPIase that is identical to the Mip-like protein of *X. campestris* pv. *campestris* 8004 was detected in the cell-free culture supernatant by two-dimensional gel electrophoresis. Thus, it is possible that, in addition to its role in virulence factor production, the *X. campestris* pv. *campestris* Mip-like protein might also act as a virulence factor that directly interacts with host cells.

By using the amino-acid sequence of *X. campestris* pv. *campestris* Mip-like protein to blast search the National Center for Biotechnology Information database, we found identical deduced proteins in *X. campestris* pv. *campestris* ATCC33913 and *X. axonopodis* pv. *citri* 306 (da Silva et al. 2002). Similar sequences have also been found in other phytopathogenic bac-

Table 3. Effects of the *mip*-like mutation on the production of exopolysaccharide^a

Strains	Exopolysaccharide yield (g/liter)
8004 (wild-type strain)	9.2 ± 0.22a
NK2699 (<i>mip</i> -like mutant)	4.3 ± 0.21b
CNK2699 (complemented strain)	9.3 ± 0.45a

^a Data are the mean ± standard deviation of triplicate measurements; different letters in each data column indicate significant differences at *P* = 0.01. Data presented were from a representative experiment and similar results were obtained in two other independent experiments.

Table 2. Bacterial strains and plasmids used in this work^a

Strains or plasmids	Relevant characteristics	Reference or source
<i>Escherichia coli</i> JM109	<i>RecA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi</i> , <i>supE44</i> , <i>relA1</i> Δ (<i>lac-proAB</i>)/F' [<i>traD36</i> , <i>lacF</i>], <i>lacZ</i> ΔM15]	Yanisch-Perron et al. 1985
<i>E. coli</i> BL21(DE3)pLysS	F [−] ompT hsdSB (rB [−] mB [−]) gal dcm (DE3) pLysS	Novagen, Darmstadt, Germany
<i>Xanthomonas campestris</i> pv. <i>campestris</i> 8004	Wild type, Rif ^r	Daniels et al. 1984
NK2699	As 8004, but <i>mip</i> -like::pK18mob, Rif ^r , Kan ^r	This work
CNK2699	NK2699 harboring pXC2699, Rif ^r , Kan ^r , Tc ^r	This work
Plasmids		
pET-30a-C(+)	Expression vector, allow the production of fusion proteins containing amino terminal 6xHis-tagged sequences. Kan ^r	Novagen
pLAFR6	Broad-host range cloning vector, Tc ^r	Huynh et al. 1989
pRK2073	Helper plasmid, Tra ⁺ , Mob ⁺ , ColE1, Spe ^r	Leong et al. 1982
pK18mob	Suicide plasmid in <i>X. campestris</i> pv. <i>campestris</i> , Kan ^r	Schafer et al. 1994
pK2699	pK18mob containing a 400-bp internal fragment of <i>mip</i> -like gene, Kan ^r	This work
pXC2699	pLAFR6 containing a 1,599-bp fragment including <i>mip</i> -like gene, Tc ^r	This work

^a Rif^r, Kan^r, Spe^r, and Tc^r = rifampicin-, kanamycin-, spectinomycin-, and tetracycline-resistant, respectively.

teria, such as *X. oryzae* pv. *oryzae* (Lee et al. 2005), *Pseudomonas syringae* pv. *tomato* (Buell et al. 2003), *P. syringae* pv. *phaseolicola* (Joardar et al. 2005), and *Erwinia carotovora* subsp. *atroseptica* (Bell et al. 2004). It is a subject of considerable interest to determine the relative contribution of these sequences to pathogenesis in their hosts. Continued progress in the understanding of Mips contribution to pathogenicity holds

out promise for mitigating infectious damage across a whole range of organisms from plants to human beings.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions.

The bacteria and plasmids used in this work are listed in Table 2. *E. coli* strains were grown in LB medium (Miller 1972) at 37°C. *X. campestris* pv. *campestris* strains were grown in NYG medium (Daniels et al. 1984) at 28°C. Antibiotics were used at the following final concentrations: rifampicin, 50 µg/ml; kanamycin, 25 µg/ml; ampicillin, 100 µg/ml; chloramphenicol, 100 µg/ml; and tetracycline, 15 µg/ml for *E. coli* and for *X. campestris* pv. *campestris*, rifampicin, kanamycin, and tetracycline at 5 µg/ml.

DNA manipulation.

DNA manipulation was performed following the procedures described by Sambrook and associates (1989). The conjugation between the *X. campestris* pv. *campestris* and *E. coli* strains was performed as described by Turner and associates (1985). Restriction enzymes and DNA ligase were used in accordance with the manufacturer's instructions (Promega, Shanghai, China).

Construction of an *E. coli* strain for production of *X. campestris* pv. *campestris* (His)₆-Mip-like protein.

A 699-bp PCR product containing *X. campestris* pv. *campestris* *mip*-like coding region was amplified, using the total DNA of *X. campestris* pv. *campestris* 8004 as template and the primer set 2699PF and 2699PR (5'-GTCGGATCCATGAAGTTGCGTTCGATCGCGGTCGCTGTG-3' and 5'-CTCAAGCTTTTACTTGACGCTGACCAGCTTGATCTCGA-3') (the underlined sequences indicate the restriction sites for *Bam*HI and *Hind*III, respectively), designed according to the ORF XC2699 sequence in the genome of *X. campestris* pv. *campestris* 8004 (Qian et al. 2005). After confirmation by sequencing analysis, the amplified DNA fragment was digested with *Bam*HI and *Hind*III and was ligated into the *Bam*HI and *Hind*III sites of the expression vector pET-30a-C(+) (Novagen, Darmstadt, Germany) (Table 2), which is designed to allow the production of a protein containing six additional His residues at the N terminus. The ligation mixture was used to transform *E. coli* BL21(DE3)pLysS (Novagen) (Table 2), and transformants were screened on LB medium with kanamycin and chloramphenicol. A transformant harboring the recombinant plasmid that carries the *mip*-like coding region without any mutation, as confirmed by DNA sequencing, was named BL21(DE3)pLysS/pET-30-2699 and was used for (His)₆-Mip-like protein production.

X. campestris pv. *campestris* (His)₆-Mip-like protein purification.

Overnight culture of BL21(DE3)pLysS/pET-30-2699 (10 ml) was used to inoculate a 200-ml LB medium with kanamycin and chloramphenicol, and the medium was cultured at 37°C with shaking at 200 rpm. When the cell concentration reached an optical density at 600 nm (OD₆₀₀) = 0.6, IPTG (isopropyl-beta-D-thiogalactopyranoside) was added to the culture at a final concentration of 1 mM, and the culture was further incubated at 28°C for 4 h. Cells were harvested by centrifugation at 4°C for 20 min at 4,000 rpm. Cells (1 g wet weight) were resuspended in 4 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole), and cells were ultrasonicated for 30 min. The cell lysate was centrifuged at 4,000 × g for 20 min at 4°C, and then, the clear supernatant was transferred into a 50-ml centrifugation tube and was mixed with 2.5 ml of pre-washed Ni-NTA resin (Qiagen, Hilden, Germany) in the lysis

Fig. 6. The *mip*-like gene is required for the full virulence and in planta growth of *Xanthomonas campestris* pv. *campestris*. **A**, Black rot symptoms caused by *X. campestris* pv. *campestris* strains on inoculated leaves of Chinese radish. Photographs were taken on day 10 postinoculation. **B**, The average lesion lengths caused by the *X. campestris* pv. *campestris* strains. Values are the mean ± standard deviation (SD) from three repeats, each with 60 leaves. **C**, The growth of bacteria in inoculated leaves. Data are the mean ± SD from three repeats. ● represents the wild-type strain 8004, △ the *mip*-like mutant NK2699, and ▲ the *mip*-like complemented strain CNK2699.

buffer. After shaking at 200 rpm at 4°C for 2 h, the batch was poured into a plastic Ni-NTA spin column (Qiagen) and then washed several times with 50 ml of precooled wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole) until the absorbance at 280 nm <0.01. Finally, the (His)₆-Mip-like protein was eluted with 500 µl of precooled elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole) and was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis or stored at -20°C.

Preparation of the extracellular and periplasmic proteins.

The extracellular and periplasmic proteins of *X. campestris* pv. *campestris* were prepared using the method described by Feilmeier and associates (2000). *X. campestris* pv. *campestris* cells were grown in NYG medium at 28°C with shaking at 200 rpm for 15 h (at the exponential phase of growth, OD₆₀₀ ≈ 1.0), and then, the bacterial cells were collected by centrifugation at 4,000 × g for 10 min and the cell-free culture supernatant was transferred to another tube and was reserved as the extracellular fraction. The cell pellet was resuspended in 0.5 ml of periplast buffer (20% sucrose, 1 mM EDTA, 30,000 U of Ready-Lyse lysozyme [Promega, Shangshai, China] per milliliter) and was incubated on ice for 5 min. Spheroplasts were then pelleted by centrifugation at 12,000 × g for 2 min, and the supernatant was reserved as the periplasmic fraction.

The total proteins in the culture supernatant and the periplasmic fraction were determined by using the Cayman Chemical protein determination kit (Cayman Chemical, Ann Arbor, MI, U.S.A.), which is based on the well-known Bradford method (Bradford 1976).

PPIase assay and inhibition test.

Protease-coupled assay (Fischer et al. 1989) was used to measure the PPIase activity of the purified (His)₆-Mip-like protein, the culture supernatant, and the periplasmic fraction. Measurements were performed in 35 mM Hepes buffer (pH 7.8) at 10°C with Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide

(Sigma-Aldrich, St. Louis) as substrate at the final concentration of 0.1 mM. α-Chymotrypsin (Sigma-Aldrich) was used to cleave the anilide bond of the *trans* (but not the *cis*) confirmation at a concentration of 0.2 mg/ml in the reaction mixture. The purified (His)₆-Mip-like protein (100 µl, 0.19 mg/ml), culture supernatant (4 mg total protein per milliliter), or periplasmic fraction (4 mg total protein per milliliter) were added to 1 ml of reaction mixture. The reaction product of chymotryptic cleavage, 4-nitroanilide, was detected by measuring the absorbance at 390 nm. The timecourse of the reaction was monitored with an Ultrospec 3300 pro spectrophotometer (Amphamacia Biotech, Biochrom Ltd., Cambridge).

Inhibition of PPIase activity was performed by adding FK506 or cyclosporin A (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) (resolved in 50% ethanol) to the PPIase activity assay reaction mixture at a final concentration of 5, 10, 15, 20, or 25 µM, respectively, and the PPIase activities of the (His)₆-Mip-like protein under these conditions were calculated and compared with those without addition of FK506 or cyclosporin A.

Construction of a mip-like gene mutant.

The *mip*-like gene XC2699 was inactivated by homologous suicide plasmid integration as described by Windgassen and associates (2000), using pK18mob as the vector (Schafer et al. 1994). A 400-bp internal fragment of the *mip*-like gene was amplified using the total DNA of *X. campestris* pv. *campestris* 8004 as template and the primer set 2699MF and 2699MR (5'-GTCCGATCCCGGTGCTGGCCTGGGTCG-3' and 5'-CTCAAGCTTGAAGTTGCGTTCGATCGCGG-3') (the underlined sequences indicate restriction sites for *Bam*HI and *Hind*III, respectively), which was designed according to the *mip*-like gene XC2699 sequence. After confirmation by sequencing, the amplified DNA fragment was cloned into the suicide plasmid pK18mob to create the recombinant plasmid pK2699. The plasmid pK2699 was transformed into *E. coli* JM109 (Yanisch-Perron et al. 1985) by transformation and then was introduced

Fig. 7. The *mip*-like gene is required for production of extracellular protease in *Xanthomonas campestris* pv. *campestris*. Overnight culture (2 µl) was spotted onto NYG (5 g of peptone, 3 g of yeast extract, and 20 g of glycerol per liter) plates containing 0.5% (wt/vol) skimmed milk (for protease activity), 0.25% (wt/vol) carboxymethylcellulose (for endoglucanase), or 0.1% (wt/vol) starch (for amylase). Plates were stained when necessary and were photographed after incubation at 28°C for 24 h (for endoglucanase and amylase activity test) or 48 h (for protease activity test). Three plates were inoculated in each experiment, and each experiment was repeated three times. Data presented were from a representative plate, and similar results were obtained in all plates of an experiment or in the plates of other independent experiments.

into *X. campestris* pv. *campestris* 8004 by triparental conjugation, using pRK2073 (Leong et al. 1982) as the helper plasmid. Transconjugants were screened on NYG supplemented with rifampicin and kanamycin, and the obtained transconjugants with a mutation in the *mip*-like gene were confirmed by PCR. Confirmation PCR was performed, using the total DNA of the transconjugants as template and the primers P18conF, 5'-GCCGATTCATTAATGCAGCTGGCAC-3', which is located in pK18mob, and 2699conR, 5'-AACTCACCTGCCCGGCGTGG-3', which is located downstream of the 400-bp internal fragment of XC2699. The expected PCR products were further confirmed by sequencing. One of the confirmed mutant transconjugants was named NK2699 (Table 2) and was chosen for further study.

Complementation of NK2699.

For complementation of the *mip*-like mutant NK2699, a 1,599-bp DNA fragment containing the entire *mip*-like gene XC2699 (from 300 bp upstream of the start codon to 600 bp downstream of the stop codon) (Fig. 1) was amplified by PCR, using the total DNA of *X. campestris* pv. *campestris* 8004 as the template and the primer set 2699Cf and 2699CR [5'-GTCCGATCCCCGCTGCCGCTCTGCAAATGC-3' and 5'-CTCAAGCTTCGCATCGGCATCGGACGGCTCG-3' (the underlined sequences indicate restriction sites for *Bam*HI and *Hind*III, respectively)]. After confirmation by sequencing, the amplified DNA fragment was cloned into pLAFR6 to generate the recombinant plasmid pXC2699. The plasmid pXC2699 was transferred into the mutant NK2699 by triparental conjugation. The transconjugants carrying pXC2699 were screened on NYG with rifampicin, kanamycin, and tetracycline. A confirmed transconjugant representative was named CNK2699 (Table 2) and was chosen for further study.

Test of extracellular enzyme activity and exopolysaccharide production.

A radial diffusion assay (Tang et al. 1987) was used to test the activity of extracellular enzyme protease, endoglucanase, and amylase. Overnight culture (2 µl, OD₆₀₀ ≈ 1.0) of each *X. campestris* pv. *campestris* strain was spotted onto a NYG plate containing 0.5% (wt/vol) skimmed milk (for protease) (Sangon, Shangshai, China), 0.25% (wt/vol) carboxymethylcellulose (for endoglucanase) (Sangon), or 0.1% (wt/vol) starch (for amylase) (Sangon), respectively, and was incubated at 28°C for 24 h (endoglucanase and amylase) or 48 h (protease). Plates were stained when necessary, as described by Tang and associates (1991). Zones of clearance around the spot due to the degradation of the substrate were photographed. Three plates were inoculated in each experiment, and each experiment was repeated three times. The relative activity of the enzyme was indicated by the diameter of the clear zone.

The exopolysaccharide production was measured as previously described, with minor modification (Wilson et al. 1998). Overnight culture (2 ml, OD₆₀₀ ≈ 1.0) of each *X. campestris* pv. *campestris* strain was used to inoculate 100 ml of NYG medium containing 4% glucose in a 500-ml flask and was kept at 28°C with shaking at 200 rpm for 5 days. The exopolysaccharide was precipitated from the culture supernatant by the addition of three volumes of 95% ethanol. The pelleted exopolysaccharide was washed with 70% ethanol and was air-dried and weighed. Three flasks were inoculated in each experiment, and each experiment was repeated three times.

Virulence assay and determination of bacterial load in planta.

Virulence was tested on potted Chinese radish growing in a greenhouse with a 12-h day and 12-h night cycle of illumina-

tion by fluorescent lamps at 25 to 28°C. Seedlings with four fully expanded leaves were used for inoculation. Bacterial cells were grown in NYG medium at 28°C with shaking at 200 rpm for 15 h (at the exponential phase of growth). The concentration of bacterial inocula was adjusted to OD₆₀₀ = 0.1. Two leaves per plant were inoculated by the leaf-clipping method (Dow et al. 2003). A total of 60 leaves were inoculated for each independent experiment, repeated three times. After maintenance in 100% humidity for 24 h, the inoculated plants were maintained in the growth conditions described above. Lesion length was measured 10 days postinoculation. The same experiment was repeated three times.

The growth of bacteria in radish leaf tissue was measured using the method previously described (Tang et al. 2005).

HR test.

The HR was tested on the pepper plant ECW-10R (*Capsicum annuum*), which is one of the nonhosts commonly used to test the HR of *X. campestris* pv. *campestris* (Castañeda et al. 2005; Newman et al. 2001). The plants were inoculated by infiltrating approximately 5 µl of bacterial culture (1 × 10⁸ CFU/ml) suspended in 10 mM sodium phosphate buffer (containing 5.8 mM NaH₂PO₄ and 4.2 mM NaH₂PO₄, pH 7.0) into the abaxial leaf surface using a blunt-end plastic syringe. The inoculated plants were maintained in a greenhouse with a 2-h day and 12-h night cycle of illumination by fluorescent lamps and a constant temperature of 28°C and were observed at 24, 36, and 48 h after inoculation. HR symptoms were photographed 48 h postinoculation. At least three plants were inoculated in each experiment, and each experiment was repeated at least two times.

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