Mutation of the gene encoding a major outer-membrane protein in Xanthomonas campestris pv. campestris causes pleiotropic effects, including loss of pathogenicity

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Xanthomonas campestris pv. campestris (Xcc) is the phytopathogen that causes black rot in crucifers. The xanthan polysaccharide and extracellular enzymes produced by this organism are virulence factors, the expression of which is upregulated by Clp (CRP-like protein) and DSF (diffusible signal factor), which is synthesized by RpfF. It is also known that biofilm formation/dispersal, regulated by the effect of controlled synthesis of DSF on cell–cell signalling, is required for virulence. Furthermore, a deficiency in DSF causes cell aggregation with concomitant production of a gum-like substance that can be dispersed by addition of DSF or digested by exogenous endo-β-1,4-mannanase expressed by Xcc. In this study, Western blotting of proteins from a mopB mutant (XcMopB) showed Xcc MopB to be the major outer-membrane protein (OMP); Xcc MopB shared over 97% identity with homologues from other members of Xanthomonas. Similarly to the rpfF mutant, XcMopB formed aggregates with simultaneous production of a gummy substance, but these aggregates could not be dispersed by DSF or endo-β-1,4-mannanase, indicating that different mechanisms were involved in aggregation. In addition, XcMopB showed surface deformation, altered OMP composition, impaired xanthan production, increased sensitivity to stressful conditions including SDS, elevated temperature and changes in pH, reduced adhesion and motility and defects in pathogenesis. The finding that the major OMP is required for pathogenicity is unprecedented in phytopathogenic bacteria.

INTRODUCTION

In Gram-negative bacteria, outer-membrane proteins (OMPs) play key roles in the structural integrity of the outer membrane (OM) and function as transporters, membrane pores, recognition proteins, membrane-bound enzymes or components of signal transduction cascades (Bishop, 2008; Koebnik et al., 2000; Kostakioti et al., 2005). The OMPs of bacterial animal pathogens are required for normal growth and resistance to stressful conditions; such proteins include OprF of Pseudomonas aeruginosa (Gotoh et al., 1989; Rawling et al., 1998; Woodruff & Hancock, 1989) and OmpA of Escherichia coli (Wang, 2002). OMPs may also play important roles in pathogenesis, for example, OspC of Borrelia burgdorferi (Pal et al., 2004), OmpA of E. coli (Khan et al., 2003; Prasadara et al., 1996; Wang, 2002), Opa and OpcA of Neisseria meningitidis (Moore et al., 2005), PagC of Salmonella typhimurium (Miller et al., 1992), Ail of Yersinia enterocolitica (Miller et al., 1989, 1990) and OmpA of Acinetobacter baumannii (Choi et al., 2008). OMPs of bacterial plant pathogens have also been shown to be involved in pathogenicity in several instances. For example, in Dickeya dadantii (Erwinia chrysanthemi), HrcC, required for a functional type III secretion system, has been implicated in virulence (Yang et al., 2002), HecA, an adhesin, is important in virulence and contributes to attachment, aggregation and epidemical cell killing (Rojas et al., 2002) and TolC is involved in resistance to antimicrobial plant chemicals and in the survival and colonization of the pathogen in plant tissues (Barabote et al., 2003). In Xanthomonas, mutation in the Xanthomonas oryzae pv. oryzae xadA gene caused a deficiency in virulence (Ray et al., 2002), and HrpA1 of Xanthomonas campestris pv.
vesiculatoria has been shown to be essential for pathogenicity in host plants and induction of a hypersensitive reaction in non-host plants (Wengelnik et al., 1996). None of these OMPs of bacterial plant pathogens has been shown to be a major OMP.

Species of the Gram-negative genus Xanthomonas, mostly plant-pathogenic organisms, can be further divided into various pathovars depending on the plant hosts infected (Dye et al., 1980; Swings & Civerolo, 1993). For example, X. campestris pv. campestris (Xcc) is the causative agent of black rot in crucifers (William, 1980). The OMPs of 54 isolates representing 16 pathovars of X. campestris have been resolved by SDS-PAGE. It was found that heterogeneity in OMP profiles existed within individual pathovars, and that a 37 kDa protein was the most abundant in all nine isolates of pv. campestris (Ojanen et al., 1993). In addition, similarly to other Gram-negative bacteria, Xcc can liberate outer-membrane vesicles (OMVs) from the OM during growth, and it was found that the OMV proteome did not contain all high-abundance OMPs (Sidhu et al., 2008). Despite these findings, none of the OMPs has been studied further, and the roles of these proteins in pathogenicity have not been investigated.

In the present study, we show that Xcc MopB is the major OMP and that mutation of the encoding gene results in motility and EPS productivity and loss of pathogenicity.

**METHODS**

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, Luria-Bertani (LB) broth or LB agar (Miller, 1972) was used to grow Xcc and E. coli at 28 and 37 °C, respectively. XOLN (Fu & Tseng, 1990) is a basal salt medium and was supplemented with 0.0625% (w/v) yeast extract and 0.0625% (w/v) tryptone. For measurements of Xcc growth, overnight cultures were diluted into 30 ml LB medium to obtain an initial OD550 of 0.35, followed by growth at 28 °C unless otherwise specified; the OD550 was measured at appropriate intervals. When the effect of pH on growth was tested, the medium was adjusted to pH 5.0–9.0 using citrate buffer and the OD550 was measured 16 h later. To test for sensitivity to SDS, the detergent was added at concentrations of up to 0.02% (w/v) and the OD550 was measured 8 h later. The following antibiotics were added when required (μg ml⁻¹): ampicillin (50), kanamycin (50), tetracycline (15), gentamicin (15).

**Enzymes and chemicals.** Restriction endonucleases were purchased from Takara Biochemicals. Taq DNA polymerase and PCR-related materials were obtained from either Merck or Takara Biochemicals. Laboratory-grade chemicals were purchased from Sigma or Merck. Synthetic diffusible signal factor (DSF; cis-11-methyl-2-dodecanoic acid) was obtained from Cayman Chemical (Ann Arbor, MI, USA).

**DNA techniques, SDS-PAGE and Western blotting.** DNA manipulations were performed as described in Sambrook & Russell (2001). Transformation of Xcc cells was achieved by electroporation (Wang & Tseng, 1992). Bacterial proteins were separated by SDS-PAGE (12 %, w/v), followed by staining of protein bands with Coomassie brilliant blue R-250. For Western blotting, proteins were transferred electrophoretically to PVDF membranes (Hybond-P; Amersham Pharmacia Biotech). After blocking, nitrocellulose sheets were incubated with primary antibody (mouse anti-MopB antiserum, AbD serotec, Kidlington, UK) and appropriate biotinylated secondary antibodies.

**Table 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>X. campestris pv. campestris</strong></td>
<td></td>
<td></td>
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<tr>
<td>Xc17</td>
<td>Virulent wild-type strain isolated in Taiwan, Ap'</td>
<td>Yang &amp; Tseng (1988)</td>
</tr>
<tr>
<td>XcMopB</td>
<td>mopB mutant derived from Xc17, Ap' Km'</td>
<td>This study</td>
</tr>
<tr>
<td>XcRpfF</td>
<td>rpfF mutant derived from Xc17, Ap' Gm'</td>
<td>Hsiao et al. (2009)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
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<tr>
<td>DH5α</td>
<td>endA1 hisdRI7 (rC m2' ) supE44 thi-1 recA1 gyrA relA1 φ80dlacZAM15 Δ(lacZYA-ArgF)U169; general cloning host</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>BL21/DE3</td>
<td>F' ompT hsdSBI (rB m2' ) gal dcm met (DE3)</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
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<tr>
<td>pOK12</td>
<td>E. coli general cloning vector derived from P15A replicon, with lacZa fragment, Km'</td>
<td>Vieira &amp; Messing (1991)</td>
</tr>
<tr>
<td>pRK415</td>
<td>Broad-host-range vector derived from RK2, with lacZa fragment, Tc'</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pET-30b</td>
<td>Expression vector, Km'</td>
<td>Novagen</td>
</tr>
<tr>
<td>pT&amp;A vector</td>
<td>PCR cloning vector, Ap'</td>
<td>Eastern</td>
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<td>pOK-mopB</td>
<td>500 bp PCR fragment amplified from mopB (nt 301–800 relative to translation start site) and cloned into pOK12</td>
<td>This study</td>
</tr>
<tr>
<td>pRK-mopB</td>
<td>1183 bp PCR fragment amplified from mopB (nt -85 to 1098 relative to translation start site) and cloned into pRK415</td>
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<td>pET-mopB</td>
<td>pET-30b derivative with 1095 bp Ndel–Xhol fragment carrying the Xc17 mopB gene</td>
<td>This study</td>
</tr>
<tr>
<td>pET-manA924</td>
<td>pET-30b derivative with 924 bp Ndel–Xhol fragment carrying the Xc17 manA gene</td>
<td>Hsiao et al. (2010)</td>
</tr>
</tbody>
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amplified fragment was ligated into introduced strain XcMopB(pRK-
mented strain XcMopB was constructed by insertion.
were used were amplified by PCR using Xc17 chromosomal DNA as the template and cloned into the broad-host-range vector pRK415. The primers used were mopB-F (5'-GAAGGCCGGCGCTGGAACCC-3') and mopB-R (5'-GCTCCAGGACGACGGTCTGAGCT-3') using Xc17 chromosomal DNA as the template. The ampiclon was ligated into the EcoRV site of the E. coli vector pRK12, which cannot be maintained in Xcc, resulting in plasmid pOK-mopB. After sequence verification, pOK-mopB was introduced into Xc17 by electroporation and transformants were selected on LB medium supplemented with kanamycin. Insertional inactivation of mopB by integration of pOK-mopB was confirmed by PCR, Southern hybridization and Western blotting (not shown).

For genetic complementation, the complete mopB gene (a 1183 bp DNA fragment; nt 85 to 1098 relative to the mopB initiation codon) was cloned by PCR using Xc17 chromosomal DNA as the template and cloned into the broad-host-range vector pRK415. The primers used were mopBC-F (5'-AACGTGGCCGCGCTGGAACCC-3') and mopBC-R (5'-TGTTCAAGTTCTGTAGGCCTG-3'). The amplified fragment was ligated into EcoRV-digested pRK12. After verification of the sequence, the insert was excised using HindIII and Xhol and ligated into pRK415. The resulting plasmid pRK-mopB was introduced into the mopB mutant XcMopB to form the complemented strain XcMopB(pRK-mopB).

Fractionation of Xcc cells. Fractionation of Xcc cells was carried out as described by Browning et al. (2003) and Tadayyon et al. (1994), with some modifications. Two 80 ml cultures of each strain, with growth commencing at OD550 0.35, were grown overnight and then centrifuged at 6000 g at 4 °C for 5 min. Cells were suspended in cold TM buffer (10 mM Tris, pH 8.0, containing 8 mM MgSO4), washed three times, resuspended in 4.0 ml of the same buffer and passed four times through an Amino French press at a pressure of 18 000 p.s.i. Unbroken cells and cell debris were removed by centrifugation at 14 000 g at 4 °C for 30 min. Supernatants were then centrifuged at 135 000 g at 4 °C for 1 h. Supernatants, containing cytoplasmic and periplasmic proteins, were retained. Pellets, containing membranes and ribosomes, were suspended in 1.0 ml cold TM buffer by gentle aspiration and ejection using a 25-gauge needle attached to a 1 ml syringe, and resuspended samples were centrifuged at 135 000 g as before. Pellets were rinsed with 1.0 ml cold TM buffer, resuspended in 3.9 ml 0.25% (w/v) Sarkosyl and loaded into 3.9 ml ultracentrifuge tubes. After incubation at room temperature for 1 h, the tubes were centrifuged at 135 000 g for 1 h, as above. Supernatants, containing the Sarkosyl-insoluble inner membrane (IM), were retained. The Sarkosyl-insoluble pellets, containing OM fractions, were washed twice with 1.0 ml 0.25% (w/v) Sarkosyl, incubated at room temperature for 1 h and centrifuged at 135 000 g as described above. The pellets, containing the OM fraction, were suspended in 40 μl cold TM buffer.

Protein concentrations in Sarkosyl-soluble and Sarkosyl-insoluble fractions were determined using a Bio-Rad detergent-compatible protein assay kit (catalogue no. 500-0012) and a regular Bio-Rad protein assay kit (catalogue no. 500-0006), respectively.

Expression and purification of recombinant MopB and preparation of antibodies against MopB. The 1095 bp DNA fragment containing the complete mopB sequence (nt 1–1095 relative to the mopB initiation codon) was amplified by PCR using Xc17 chromosomal DNA as the template and primers mopB-BndF (5'–CATATGAAACAGAAAAACTCTACCTGCGC-3') and mopBXhol-R (5'-CTCGAGGTTCTGGACGCTAGTC-3'), with the underlined nucleotides representing novel NdeI and XhoI recognition sites, respectively. The ampiclon was cloned using an ECOS TA cloning kit (Yeolast). The cloned mopB-containing DNA fragment, after confirmation of identity by sequencing, was excised using NdeI and XhoI and ligated into pET30b. The resulting plasmid pET-mopB was introduced into E. coli strain BL21(DE3) for expression of recombinant MopB, which had six His residues attached to the C terminus.

Cells of E. coli BL21(DE3) (pET-mopB) were grown at 28 °C with shaking (150 r.p.m.) to an OD600 of 1.0, isopropyl β-D-thiogalacto-
oside (IPTG) was added to a final concentration of 0.25 mM and growth was continued for 4 h at 28 °C. Cells were harvested by centrifugation at 10 000 g for 15 min, resuspended in buffer A (20 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl, 5 mM imidazole, 6 M urea and 1 mM PMSF) and sonicated on ice. Unbroken cells and cell debris were removed by centrifugation at 12 000 g for 30 min. The supernatant was filtered through a 0.45 μm membrane filter and applied to a column (Bio-Rad Econo-Pac) containing Ni2+-nitrilotriacetic acid-Sepharose Fast flow resin (Amersham Bioscience) for affinity binding. The column was washed with buffer B (20 mM Tris/HCl, pH 8.0, containing 0.5 M NaCl, 40 mM imidazole and 6 M urea). Recombinant MopB bound to the resin was eluted from the column by 300 mM imidazole. Recombinant MopB protein was employed to immunize mice by subcutaneous injection at 2-week intervals. Antiserum was collected after four boosts.

Expression and purification of recombinant endo-1,4-β-
mannanase. The plasmid pET-manaA924, carrying a cloned manA gene encoding the mannanase of Xcc, was the kind gift of Dr Yi-Min Hsiao (Central Taiwan University of Science and Technology). Expression and purification of recombinant mannanase was achieved as described by Hsiao et al. (2010). The quantitative endo-1,4-β-mannanase assay employed the Megazyme procedure, using synthetic aso-corab-galactomannan as the substrate (Megazyme).

Plate assay for hydrolytic activities. To test for cellulase, protease, amylase, pectinase and mannanase activities of Xcc cells, indicator plates were prepared containing XOLN or LB medium supplemented with 0.5% (w/v) CM-cellulose, 1% (w/v) skimmed milk, 1.5% (w/v) soluble starch, 1% (w/v) polygalacturonic acid or 0.1% (w/v) locust bean gum. Cells were washed and adjusted to an OD550 of 1.0 with LB medium and 3 μl aliquots were spotted onto the plates, followed by incubation at 28 °C for 48 h. Plates were stained as described previously (Bourgault & Bewley, 2002; Tang et al., 1991).

Estimation of xanthan polysaccharide levels. The levels of xanthan polysaccharide were measured using a procedure described previously (Fu & Tseng, 1990), with some modifications. Cells from an overnight culture were harvested by centrifugation and inoculated at an initial OD550 of 0.35 into XOLN medium containing 2% (w/v) glucose. After 72 h, cultures were diluted 2- to 10-fold with distilled water and centrifuged at 12 000 g for 20 min to remove cells. The xanthan polysaccharide in the supernatant was precipitated by addition of NaCl to 40 mM and ethanol to 70% (v/v) and overnight incubation at −20 °C. The suspension was centrifuged at 20 000 g for 30 min. The pellet was washed once with 70% (v/v) ethanol and resuspended in distilled water. Xanthan levels were determined using
a modified anthrone method (Lin & Tseng, 1979) and calculated with reference to a standard curve constructed using purified xanthan polysaccharide.

**Pathogenicity test.** To test for pathogenicity of Xcc, 2-week-old potted cabbage seedlings (variety Big Star; Known-You Seed Co., Kaohsiung, Taiwan) were used in a procedure that has been described previously (Yang & Tseng, 1988). Leaves to be inoculated were wounded by cutting (length of cut approx. 1 cm) with a pair of scissors at the edge at an angle of about 45 degrees to the midrib. Bacterial cells from an overnight culture were diluted 50-fold into fresh LB broth and grown to an OD	extsubscript{550} of 1.0. Aliquots (10 µl) of each culture were inoculated by spreading onto the cut surfaces. Inoculated seedlings were kept in a growth chamber at 28 °C with constant illumination. Lesions on leaves were observed until 14 days post-inoculation for each strain tested. Testing was conducted in three independent experiments, each with six replicates.

**Assays of abiotic and biotic adhesion and motility.** A quantitative assay for adhesion of Xcc strains was performed as described previously (Chao et al., 2008; Jackson et al., 2002). Cells from overnight cultures (100 µl aliquots, OD	extsubscript{550} 1.0) were inoculated into 3.0 ml XOLN medium [containing 2% (w/v) glucose] in 20 ml Universal tubes (QingFa) and incubated with shaking at 28 °C for 24 h. Cells that bound to the glass surface were measured by removing the medium, rinsing the tubes with 3 ml distilled water (three times) and staining bound cells with 2 ml 0.3% (w/v) crystal violet (Bio Star). The free dye was removed and the tubes were rinsed three times with distilled water. Next, bound crystal violet was solubilized in 1 ml 33% (v/v) acetic acid and the A	extsubscript{630} was determined using a Hitachi U-1900 spectrophotometer.

The procedures used for the biotic adhesion assay were as described by Rigano et al. (2007) with some modifications. Cabbage leaf discs of approximately 3.8 cm	extsuperscript{2} were cut from healthy leaves and placed in a flask, to which 30 ml bacterial cells in XOLN medium containing 2% (w/v) glucose at OD	extsubscript{550} of 0.1 was added, and incubated at 28 °C. After 3, 12 and 24 h of incubation, the leaf discs were rinsed gently three times with distilled water. Adherent bacteria on the leaf surface were warmed at 60 °C for 20 min and stained with 3 ml 0.30% (w/v) crystal violet (Bio Star). The free dye was removed and the tubes were rinsed three times with distilled water. Next, bound crystal violet was solubilized in 1 ml 33% (v/v) acetic acid and the A	extsubscript{630} was determined using a Hitachi U-1900 spectrophotometer.

**Motility of Xcc cells was assayed by a method described previously (Lee et al., 2003). Cells from overnight cultures were washed and diluted with LB medium to an OD	extsubscript{550} of 1.0 and 3 µl aliquots of cells were inoculated onto XOLN plates containing 0.3 or 0.5% (w/v) agar to test for swimming and swarming motility, respectively. Motility was assessed qualitatively by measuring the diameter of the circular halo formed by bacterial cells on plates incubated at 28 °C for 3 days.**

**Electron microscopy.** Bacteria incubated in LB broth were washed with PBS and fixed overnight with 2% (w/v) glutaraldehyde, 4% (w/v) paraformaldehyde and 1% (w/v) tannic acid in 0.1 mol cacodylate buffer 1–1, pH 7.4. After washing with PBS, cells were stained and prepared as described by Lin et al. (2007). Treated cells were viewed in a JEOL T300 electron microscope.

**RESULTS**

**MopB is highly conserved in Xanthomonas**

In the nearly completed genomic sequence of Xcc strain 17 (Xc17), the model strain used in our laboratory, a gene has been assigned as encoding MopB (the major OMP; http://xcc.life.nthu.edu.tw/). The MopB protein deduced from the gene (1098 bp) contains 365 amino acids, with an N-terminal signal peptide of 22 amino acids. Removal of the signal peptide would produce a mature protein of 37268 Da with a pI of 4.50.

Complete genome sequences are available for three strains of Xcc. Our sequence comparison indicated that the Xc17 MopB was identical in both size and amino acid sequence to MopBs from all sequenced Xcc genomes (GenBank accession numbers NP_636321, YP_244364 and YP_001904823). The Xc17 MopB was closely related (more than 97% identity) to MopB from other members of Xanthomonas, including *Xanthomonas axonopodis* pv. citri 306 (NP_641359), *X. campestris* pv. vesicatoria 85-10 (YP_362773), *X. oryzae* pv. oryzicola BLS256 (ZP_02242092) and *X. oryzae* pv. oryzicola PX099A and MAFF 311018 (YP_001915346 and BAE70245). The Xc17 MopB showed a high level of identity with homologues from *Stenotrophomonas maltophilia* R551-3 and K279 (87%; YP_002027188 and YP_001970833) and four strains of *Xylella fastidiosa* (68–71%); NP_297633, NP_779898, YP_001776390 and YP_001830479). Xc17 MopB shared lower degrees of identity with (i) OprF from strains of *Pseudomonas*, including *Pseudomonas putida* KT2440 and GB-1, *P. syringae* pv. tomato DC3000, *P. syringae* pv. phaseolicola 1448A and *P. aeruginosa* PA01 (28–30%; NP_744239, YP_001667841, NP_792118, YP_274287 and NP_250468); (ii) OmpF from strains of *Pseudomonas*, including *P. putida* W619 and *P. fluorescens* Pf0-1 (29.3–29.8%; YP_001748481 and YP_347509); (iii) thrombospondin type 3 repeat from *Nautilia profundicola* AmH, *P. syringae* pyrugged pyranged B728a and *Azotobacter vinelandii* AVOP (24–28%; YP_002606840, YP_235178 and ZP_00415173); (iv) Tbd0127 from *Thiobacillus denitrificans* ATCC 25259 (28.2%; YP_313885); (v) MopB from *Methyllobacoccus capsulatus* Bath (28.1%, YP_115490); (vi) outer-membrane fibronectin-binding protein from *Campylobacter fetus* subsp. fetus 82-40 and *Arcobacter butzleri* RM4018 (27%; YP_891591 and YP_001489425); and (vii) *E. coli* APEC01 OmpA (21%, YP_852080). Notably, among these homologues of Xcc MopB, only *E. coli* OmpA has been shown to be involved in pathogenesis (Khan et al., 2003; Wang, 2002). A phylogenetic tree constructed on the basis of multiple sequence alignment reflected the evolutionary relationships among these homologous proteins (Fig. 1). Notably, the relatedness among MopB homologues, displayed by the phylogenetic tree, was similar to that of the 16S rRNA gene (not shown).

Our sequence analysis also showed that these MopB homologues, belonging to the OmpA family, each had an OmpA domain NX\textsubscript{2}LSX\textsubscript{2}RAAX\textsubscript{2}VX\textsubscript{L} (de Mot & Vanderleyden, 1994a) and a calcium-binding motif DxDxDG (de Mot & Vanderleyden, 1994b; Rigden & Galperin, 2004), located in Xc17 MopB at aa 301–316 and aa 216–221, respectively.
MopB is the most abundant OMP in Xc17, and a MopB deficiency causes changes in the OMP composition

For further study of the mopB gene, a mopB mutant (XcMopB) was constructed. This mutant and the parental Xc17 were used for comparison of OMP profiles and to verify that MopB was indeed the major OMP. Membrane fractionation was based on the differential solubility of the inner membrane (IM) and OM in Sarkosyl (0.25 %, w/v), with the former being soluble and the latter insoluble in the detergent solution. Following fractionation, samples were subjected to separation by SDS-PAGE, followed by detection of MopB with antibodies prepared by immunizing mice with a recombinant MopB expressed in E. coli. To demonstrate the efficiency of fractionation in these experiments, the same samples were treated in parallel and probed with antibodies prepared against Xc17 superoxide dismutase (SOD), a protein that is confined to the cytosol. The results indicated that the SOD protein was indeed detected only in crude extracts and in fractions containing cytoplasmic and periplasmic proteins (not shown).

As shown in Fig. 2(a), the OM fraction from Xc17 displayed more than 10 distinct protein bands upon staining of gels with Coomassie brilliant blue, with a 37 kDa protein being the most abundant (lane 4). This molecular size was similar to that of MopB reported for Xcc strains 528 and 1648 (Ojanen et al., 1993) and to that calculated from the amino acid sequences of MopB predicted from Xanthomonas genomes. In the OM fraction of the mopB mutant, the 37 kDa band was missing, and more than 17 protein bands were observed, of which five were too faint to be visible in wild-type Xc17 (Fig. 2a, lanes 4 and 5). These results indicated that the OMP profile was altered by mutation of mopB. Western-blot results showed that most of the 37 kDa protein of Xc17 was localized in the OM fraction, and only very small amounts were seen in other fractions (Fig. 2b, lanes 1–4). No corresponding hybridization signal was observed from the OM fraction prepared from XcMopB (Fig. 2b, lane 5), confirming that insertional mutation had destroyed expression of the mopB gene. These data together indicated that MopB, being the most abundant protein, was the major OMP of Xcc.

Mutation in Xcc mopB causes loss of pathogenicity

Xcc is an important plant pathogen and its pathogenicity has been the focus of our research. To determine whether mutation of mopB caused loss of pathogenicity, XcMopB was used to infect cabbage seedlings. In these tests, the tissue around the leaf margin where Xc17 cells had been inoculated turned yellow at about 4 days after infection and then, at day 6, V-shaped chlorotic lesions began to spread from the leaf margin toward the midrib. The progression of symptom development in the complemented strain, XcMopB(pRK-mopB), was similar to that of Xc17. Fig. 3 shows the symptoms caused by wild-type Xc17 and the complemented strain at 10 days after inoculation. However, no symptoms were visible on leaves inoculated with XcMopB, even after prolonged incubation for more than 14 days (Fig. 3). These results demonstrate clearly that XcMopB had lost the ability to cause black rot in cabbage.

XcMopB forms aggregates when cultures enter stationary phase and is more sensitive to stressful conditions than is the parental strain

During characterization of the mutant XcMopB, the effects of the mutation on bacterial growth were evaluated and several defects were identified.
(i) XcMopB forms cell aggregates. As soon as XcMopB was isolated, we noticed that cells formed aggregates after overnight incubation, although bacteria of the parental Xc17 strain remained dispersed. XcMopB cells grew at the same rate as the wild-type strain before entering stationary phase (i.e. prior to 14 h) and both reached an OD550 of about 3.7. The OD550 of Xc17 continued to increase slightly until saturation (OD550 4.4) at 22 h, whereas XcMopB cultures ceased to grow at 14 h and began to decrease in bacterial numbers (as detected by colony counting) at 16 h owing to formation of cell aggregates (Fig. 4). To estimate the proportion of cells in aggregates, cultures were filtered through Whatman No.1 filter paper (55-mm diameter discs) and the OD550 of the filtrates was measured. The results indicated that the OD550 of the XcMopB culture was reduced to about 69.5 % that of wild-type cells (Fig. 4), indicating that more than 30 % of the cells had become aggregated.

(ii) Aggregate formation by the mopB mutant and an rpfF mutant involves different mechanisms. Xcc rpfF mutant cells, which are deficient in the synthesis of DSF, become aggregated when cultures enter stationary phase and synthesize a gum-like substance that forms a thick

**Fig. 2.** Identification of MopB as the major OMP of Xc17 by Western blotting. Membrane fractions were prepared from wild-type Xc17 and the mopB mutant XcMopB by treatment with 0.25 % (w/v) Sarkosyl and separated into detergent-soluble (IM) and -insoluble (OM) fractions. Proteins from each fraction were separated on 12 % (w/v) SDS-polyacrylamide gels (a) followed by Western blotting using polyclonal antibodies against MopB (1:100 000) (b). Lanes: M, size markers; 1, Xc17 crude extract; 2, cytoplasm and periplasm of Xc17; 3, Xc17 IM; 4, Xc17 OM; 5, XcMopB OM. Arrowheads indicate bands in the XcMopB OM that were not observable in the Xc17 OM.

**Fig. 3.** Pathogenicity of Xcc strains. Cells (about 5x10^6 cells in 10 μl) of the wild-type Xc17, the mopB mutant (XcMopB) and the complemented strain XcMopB(pRK-mopB) were inoculated into cuts on the edges of leaves of 2-week-old potted cabbage plants. Appearance of symptoms was recorded on days 10 and 14 post-inoculation. LB, LB broth used as a control.

**Fig. 4.** Growth of Xcc strains in LB medium. Cells of the wild-type Xc17 and XcMopB were diluted into 30 ml LB medium to OD550 0.35 and grown at 28 °C. The OD550 was monitored during growth. After cultures entered stationary phase, duplicate samples were taken and one set (indicated by asterisks) was filtered prior to reading of OD550 to estimate the proportion of cells in aggregates; the other sample was read directly.
layer on the top of pelleted cells after centrifugation; addition of either DSF or purified endo-β-1,4-mannanase encoded by manA of the same bacterium can disperse the aggregates (Dow et al., 2003). In the present study, to compare aggregate properties, cultures of XcMopB and the rpfF mutant were grown in parallel. As shown in Fig. 5(a), cells of Xc17 remained dispersed throughout growth (flask 1); in contrast, XcMopB formed conspicuous clumps and sedimentation of cells was observed after 24 h (Fig. 5a, flask 2). The aggregate-forming phenotypes of the mopB and rpfF mutants were found to be similar, except that the clumps of XcMopB were larger (Fig. 5a, flask 2; Fig. 5b, flask 2). No visible gummy substance was produced by Xc17, with no obvious layer seen on the top of pelleted cells after centrifugation (10 000 g, 10 min) of 24 h cultures (Fig. 5c, tube 1); in contrast, a thick layer of gummy substance was found over the top of pelleted XcMopB (Fig. 5c, tube 2) and rpfF mutant (Fig. 5d, tube 2) cells. The pelleted gummy substance from the mopB mutant was more compact than that of the rpfF mutant, indicating that the texture and composition of these two types of gummy substance were not the same. Addition of synthetic DSF (final concentration, 10 μM) at 16 h of growth could disperse rpfF mutant cells 8 h after treatment (Fig. 5b, flask 3), but the same addition did not disperse XcMopB cells (Fig. 5a, flask 3). When purified endo-β-1,4-mannanase (5 mU; Mnase) was added to cultures after 16 h of growth, the aggregates formed by the rpfF mutant were dispersed at 0.5 h after the treatment (Fig. 5b, flask 4), but XcMopB aggregates were not dispersed (Fig. 5a, flask 4). Furthermore, treatments that blocked formation of the gum-like substance by the rpfF mutant effectively (Fig. 5d, tubes 3 and 4) could not inhibit formation of the gummy substance found in the XcMopB cultures (Fig. 5c, tubes 3 and 4). These results indicate that, although the phenotypes of the aggregates were similar, both the mechanisms involved in aggregate formation and the chemical nature of the gummy substances differed.

A dispersed growth mode, similar to that of Xc17, was restored to the complemented strain by provision of the cloned wild-type mopB gene (Fig. 5a, flask 5). No gummy substance was observed on the top of pelleted complemented cells (Fig. 5c, tube 5). These results confirmed that deficiency in MopB was indeed the factor causing cell aggregation.

(iii) XcMopB cannot grow at 37 °C. For temperature-sensitivity tests, cells were grown at either 28 °C, the normal temperature for Xcc growth, or 37 °C. At 28 °C, XcMopB grew at a similar rate to wild-type Xc17, as has been shown above (Fig. 4). When the temperature was increased to 37 °C, the growth rate of Xc17 was reduced markedly, and no significant growth of XcMopB was observed (Fig. 6a).

(iv) XcMopB cannot grow at pH higher than 8.5. To test for effects of pH on growth of the mopB mutant, cells were added to LB at 28 °C for 16 h and treated by addition of DSF (final concentration, 10 μM), purified endo-β-1,4-mannanase (5 mU; Mnase) or LB medium (–). Photographs were taken 8 h after treatment. (b) Cells of Xc17 and the rpfF mutant were treated as described in (a). (c, d) Microcentrifuge tubes containing pelleted cells of cultures from the flasks of (a) and (b), respectively. In (a) and (b), the upper photographs in each pair were taken from the front, whereas the lower ones were taken from below.
grown at different pH values after adjustment with citrate buffer. As shown in Fig. 6(b), both the mopB mutant and the parental Xc17 strain showed the best growth at pH 7.0, but, when the pH was varied, different levels of growth inhibition were observed. At pH 8.5, no significant growth of the mopB mutant was detected, whereas growth of Xc17 was about 67% of that seen at pH 7.0.

(v) XcMopB is more sensitive to SDS. It was found that Xc17 was able to tolerate SDS at 0.002% (w/v), but the final yield of cells decreased with increasing SDS concentration (Fig. 6c). Tolerance of the mopB mutant to SDS decreased markedly with increasing concentrations of SDS, and no cell growth was detectable when the concentration was increased to 0.02% (w/v), whereas Xc17 still produced about 15% of the maximum cell yield.

EPS production is impaired in the XcMopB strain
Colonies of XcMopB were less mucoid on either LB or XOLN agar containing glucose, compared with colonies of wild-type Xc17. Formation of non-mucoid colonies suggested that XcMopB produced less EPS xanthan gum than did the wild-type strain. To assess the ability of XcMopB to produce EPS, Xcc cells were grown in XOLN medium containing 2% (w/v) glucose for 72 h and EPS contents in culture supernatants were measured. The results indicated that XcMopB produced about 2044 ± 140 μg EPS ml⁻¹, approximately 58% of that produced by wild-type Xc17 (3242 ± 203 μg ml⁻¹). The complemented strain, XcMopB(pRK-mopB), produced 2849 ± 256 μg EPS ml⁻¹, about 90% of that produced by Xc17. These results indicate that the ability to synthesize EPS was impaired by mutation of the mopB gene.

Extracellular enzymes, in addition to EPS, have been implicated as virulence factors (Chan & Goodwin, 1999; Chou et al., 1997; Dharmapuri & Sonti, 1999; Dow et al., 2003; Katzen et al., 1998); hence, the hydrolytic activities of XcMopB were assayed on XOLN and LB agar plates containing skimmed milk, CM-cellulose, pectin, mannann or starch. No significant differences were found between the parental Xc17 and XcMopB.

Mutation in mopB causes a drastic change in cell-surface properties and reductions in adhesion and motility
The formation of cell aggregates by XcMopB and associated changes in OMP composition indicated that significant alterations might have occurred on the cell surface. To investigate this, cross-sections of Xcc cells were prepared and subjected to electron microscopic observation. The results showed that cells of wild-type Xc17 displayed an electron-dense cytoplasm, a less-dense IM and a surface OM (Fig. 7a). These layers were in intimate contact. In contrast, the OM of the mopB mutant appeared less compact and was deformed, with a wide electron-transparent
region that probably represents an enlarged periplasmic space (Fig. 7b). The complemented strain had a surface structure similar to that of wild-type cells (Fig. 7c). These data indicated that mutation in Xcc \textit{mopB} led to marked changes in the OM, leading to a looser connection between the IM and OM.

After determining that the outer layers of XcMopB bacteria had been altered markedly by mutation, we investigated whether the adhesion and motility properties of XcMopB were affected. Adhesion to a glass surface was assayed quantitatively by the crystal violet staining method. The results showed that, although wild-type Xc17, mutant XcMopB and the complemented strain XcMopB(pRK-\textit{mopB}) exhibited similar growth rates under our assay conditions, the mutant cells had reduced adhesion ability. The $A_{630}$ measured in samples prepared from XcMopB cells was approximately 0.16, which was about 34 % that of Xc17 ($A_{630}$ 0.47); about 85 % of adhesion ability was restored by complementation (Fig. 8a). Bacterial adhesion to cabbage leaves was tested according to the procedures described by Rigano et al. (2007) with some modifications. The results showed that the number of attached bacteria was significantly greater on discs inoculated with Xc17 and XcMopB(pRK-\textit{mopB}) than the mutant after 12 h of incubation, and the difference became even more obvious after 24 h (Fig. 8b).

Swimming and swarming were tested by inoculating Xcc cells into XOLN medium containing 0.3 and 0.5 % (w/v) agar, respectively. The colony diameters of Xc17 and XcMopB strains were about 16.6 and 12.4 mm in the swimming mode and 17.8 and 12.0 mm when swarming was permitted, respectively, 25 and 34 % less than exhibited by wild-type cells.

**DISCUSSION**

In the present study, bioinformatic analysis revealed that protein XCC0935 in the nearly completed genomic sequence of Xc17 was annotated as MopB, the major OMP B. Identification of this protein as the major OMP (the most abundant OMP) was confirmed by SDS-PAGE followed by Western blotting of OM fractions, in which a MopB band of 37 kDa, similar to the size predicted from the nucleotide sequence, was absent from the XcMopB strain constructed by insertional mutation and restored in a complemented strain providing the cloned wild-type \textit{mopB} gene \textit{in trans}. Previous SDS-PAGE analysis of OMPs from \textit{X. campestris} showed that, although heterogeneity in OMP profiles existed within individual pathovars, a 37 kDa protein was consistently the most abundant in isolates of Xcc (Ojanen \textit{et al.}, 1993). These findings are consistent with our bioinformatic analysis showing that MopB is highly conserved among members of \textit{Xanthomonas}. The Xcc MopB also has high identity with MopBs from members of the related genera \textit{Stenotrophomonas} (86.7 %) and \textit{Xylella} (68.4 %), but showed a low level of identity with OprF of \textit{P. aeruginosa} (30.3 %) and OmpA of \textit{E. coli} (20.8 %).

Mutation in the Xcc \textit{mopB} gene resulted in a marked alteration in both the composition and architecture of the OM. In our SDS-PAGE analysis, at least five OMPs were shown to be abundant in the mutant; these proteins were too faint to be visible in samples prepared from wild-type Xc17, indicating that mutation in \textit{mopB} caused alterations in the OMP profile and probably increased expression of some otherwise minor OMPs. A similar situation was found in a mutant deficient in OmpJ, a putative porin required for metal respiration of \textit{Geobacter sulfurreducens}; marked changes in the amounts of several proteins, including a membrane-associated cytochrome, were observed (Afkar \textit{et al.}, 2005). Further studies are needed to identify these proteins in Xcc and to elucidate the significance of the change in their amounts. The changes in OMP composition of the \textit{mopB} mutant were accompanied by deformation of the mutant surface, as revealed by electron microscopy. The marked change in the surface layer is probably the reason for the multiple defects observed in...
XcMopB, including formation of cell aggregates, reduced xanthan production, reduced adhesion and motility, increased sensitivity to stress conditions and loss of pathogenicity.

It is well known that mutations in Xcc rpfF, the gene responsible for the synthesis of DSF, cause formation of cell aggregates when cultures enter stationary phase (Dow et al., 2003). Upon centrifugation of aggregated cultures, a thick layer of gum-like material can be seen on top of pelleted cells. The aggregates can be dispersed by addition of either DSF or an endo-β-1,4-mannanase encoded by Xcc, indicating that the gum-like substance contains chemical bonds cleavable by endo-β-1,4-mannanase (Dow et al., 2003). In the mopB mutant, aggregated cells could not be dispersed by addition of either DSF or endo-β-1,4-mannanase, indicating that the gummy substance has a chemical structure different from the gum formed by the rpfF mutant. Thus, it appeared that aggregate formation by the mopB and rpfF mutants involved different mechanisms.

Xcc is capable of producing large amounts of xanthan polysaccharide and an array of extracellular enzymes, which collectively have long been considered important virulence determinants (Chan & Goodwin, 1999; Chou et al., 1997; Dharmapuri & Sonti, 1999; Dow et al., 2003; Dums et al., 1991; Yang & Tseng, 1988). In the present study, we have shown that production of xanthan was impaired by mopB mutation but that synthesis of extracellular enzymes was not affected. The biosynthesis of xanthan involves synthesis of sugar nucleotide precursors, formation of pentasaccharide repeating units from sugar nucleotides on a glycosyl lipid carrier, acetylation and pyruvylation of some mannose residues, polymerization of the pentasaccharide repeating units and secretion of the polymeric molecules (Katzen et al., 1998; Vorholter et al., 2008). Proteins involved in xanthan synthesis, except those involved in formation of sugar nucleotides, are encoded by the gum operon containing gumB to gumM. Functions of particular proteins in repeating unit synthesis (GumDMHKI), acetylation (GumFG) and pyruvylation (GumL) have been confirmed experimentally (Becker et al., 1998; Katzen et al., 1998; Vorholter et al., 2008). In addition, a mechanistic model proposed on the basis of data obtained from genome annotation studies suggested that GumJ flips the lipid carrier-linked pentasaccharide repeating unit to the outer face of the IM, where polymerization is performed by GumE; upon contact with the IM-associated GumC and the OM-associated GumB, a complex periplasm-spanning pore is opened to export the xanthan polymer to the milieu (Vorholter et al., 2008). As the cell surface is greatly deformed, the mopB mutation may have drastically changed the topology of GumB; without proper positioning of this important protein, the xanthan export machinery will malfunction. It is known that xanthan production is upregulated by DSF and Clp (He et al., 2006, 2007; Slater et al., 2000), but little is known about the roles of other possible regulators. In E. coli K1, expression of type 1 fimbriae, controlled by phase variation, is decreased upon deletion of ompA (Teng et al., 2006). Thus, the possibility exists that, in Xcc, MopB

**Fig. 8.** Adhesion test of Xcc strains. (a) Overnight culture (100 μl aliquots, OD550 1.0) samples of each strain were inoculated into 3.0 ml XOLN medium [supplemented with 2 % (w/v) glucose] in 20 ml Universal tubes and incubated at 28 °C for 24 h. Samples were treated as described in Methods, followed by measurement of the amount of bound crystal violet. (b) Bacterial adhesion to cabbage leaves was assayed by staining the attached bacterial cells with crystal violet at 3, 12 and 24 h post-incubation as described in Methods.
regulates the expression of the gum operon via an unidentified mechanism, although phase variation may not necessarily be involved. The pathogenesis of black rot by Xcc involves multiple steps, including at least leaf attachment, epiphytic fitness, entry, colonization, biofilm formation/dispersal and bacterial spread to the vascular system (Chan & Goodwin, 1999; Dow et al., 2003; Rigano et al., 2007). The consequence of mutation in the Xcc mopB gene is pleiotropic, including formation of cell aggregates, reduction in xanthan production, adhesion and motility and increased sensitivity to stress conditions. Since we have previously shown that a mutation causing reduction in xanthan (about 95 % reduction) resulted in only a delayed appearance of the black rot symptoms and not a complete loss of pathogenicity (Chou et al., 1997), a 42 % reduction in xanthan production is unlikely to be the major reason for the loss of pathogenicity in XcMopB. In addition, as synthesis of extracellular enzymes was not altered in this mutant, the importance of such enzymes in the initial stages of pathogenesis may be limited. Therefore, it seems reasonable to conclude that the loss of pathogenicity in XcMopB may result from a concerted effect of increased sensitivity to stress conditions, which may reduce epiphytic fitness, and aggregate formation and reduction in motility and adhesion, which would affect biofilm formation/dispersal and limit bacterial colonization and spread. Our findings of pleiotropic effects caused by mutation of the mopB gene, including loss of pathogenicity, may help the development of new strategies for the control of black rot disease.

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