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.

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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, OmpC of *Borrelia burgdorferi* (Pal *et al.*, 2004), OmpA of *E.*

coli (Khan *et al.*, 2003; Prasadarao *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 epidermal 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.

Abbreviations: Clp, CRP-like protein; DSF, diffusible signal factor; IM, inner membrane; OM, outer membrane; OMP, outer-membrane protein; Xcc, *Xanthomonas campestris* pv. *campestris*.

vesicatoria 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 pleiotropic effects, including changes in cell-surface architecture, formation of cell aggregates, reduced adhesion, 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-dodecenoic 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,

Table 1. Bacterial strains and plasmids used in this study

Ap^r, Ampicillin resistance; Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance.

Strain or plasmid	Relevant genotype or characteristics	Reference or source
<i>X. campestris</i> pv. <i>campestris</i>		
Xc17	Virulent wild-type strain isolated in Taiwan, Ap ^r	Yang & Tseng (1988)
XcMopB	<i>mopB</i> mutant derived from Xc17, Ap ^r Km ^r	This study
XcRpff	<i>rpff</i> mutant derived from Xc17, Ap ^r Gm ^r	Hsiao <i>et al.</i> (2009)
<i>E. coli</i>		
DH5α	<i>endA1 hsdR17(r_k⁻ m_k⁺) supE44 thi-1 recA1 gyrA relA1 φ80dlacZΔM15 Δ(lacZYA–argF)U169</i> ; general cloning host	Hanahan (1983)
BL21/DE3	F ⁻ <i>ompT hsdSB(r_B⁻ m_B⁻) gal dcm met</i> (DE3)	Novagen
Plasmid		
pOK12	<i>E. coli</i> general cloning vector derived from P15A replicon, with <i>lacZα</i> fragment, Km ^r	Vieira & Messing (1991)
pRK415	Broad-host-range vector derived from RK2, with <i>lacZα</i> fragment, Tc ^r	Keen <i>et al.</i> (1988)
pET-30b	Expression vector, Km ^r	Novagen
yT&A vector	PCR cloning vector, Ap ^r	Yeastern
pOK- <i>mopB</i>	500 bp PCR fragment amplified from <i>mopB</i> (nt 301–800 relative to translation start site) and cloned into pOK12	This study
pRK- <i>mopB</i>	1183 bp PCR fragment amplified from <i>mopB</i> (nt –85 to 1098 relative to translation start site) and cloned into pRK415	This study
pET- <i>mopB</i>	pET-30b derivative with 1095 bp <i>NdeI</i> – <i>XhoI</i> fragment carrying the Xc17 <i>mopB</i> gene	This study
pET-manA924	pET-30b derivative with 924 bp <i>NdeI</i> – <i>XhoI</i> fragment carrying the Xc17 <i>manA</i> gene	Hsiao <i>et al.</i> (2010)

diluted 1:100 000) and next with alkaline-phosphatase-conjugated anti-mouse IgG antibody (Promega) (diluted 1:5000), used as the secondary antibody. Reactive proteins were detected using BCIP/NBT substrate solution (PerkinElmer Life and Analytical Sciences).

Sequence analysis. Multiple alignment of protein sequences was carried out using CLUSTAL W, a dendrogram was constructed using TreeView (Page, 1996) and signal peptides were predicted using the SignalP 3.0 program from ExPASy (<http://www.cbs.dtu.dk/services/SignalP/>). Conserved OmpA domains were identified employing the CDD program from NCBI (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>).

Construction of a *mopB* mutant and a complemented strain. A *mopB* mutant of Xc17, XcMopB, was constructed by insertional mutation. A 500 bp DNA fragment internal to Xc17 *mopB* (nucleotide positions 301–800 relative to the *mopB* initiation codon) was amplified by PCR with primers *mopB*-F (5'-GAAGCCGCGGC-TGGAACCC-3') and *mopB*-R (5'-GCATCCGACGCAGGGTCTGACT-3') using Xc17 chromosomal DNA as the template. The amplicon was ligated into the *EcoRV* site of the *E. coli* vector pOK12, 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 amplified 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'-AAGCTTGCCCCGATGGCTCTGG-3') and *mopBC*-R (5'-TCTAGATTAGTTCTGGACGTTTCAGCTCG-3'). The amplified fragment was ligated into *EcoRV*-digested pOK12. After verification of the sequence, the insert was excised using *HindIII* and *XbaI* 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 Aminco 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-soluble 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 *mopBNdeI*-F (5'-CATATGAACAAGAAAATTCTCACTGCCG-3') and *mopBXhoI*-R (5'-CTCGAGGTTCTGGACGTTTCAGCTC-3'), with the underlined nucleotides representing novel *NdeI* and *XhoI* recognition sites, respectively. The amplicon was cloned using an ECOS TA cloning kit (Yeastern). 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-thiogalactopyranoside (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⁺-chelating column 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 using 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-*manA*924, carrying a cloned *manA* gene encoding the mannanase of Xc17, 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 azo-carob-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 OD₅₅₀ of 1.0. Aliquots (10 µl) of each culture were inoculated by spreading onto the cut surfaces. Infected 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, OD₅₅₀ 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 A₆₃₀ 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 cm² 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 OD₅₅₀ 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 for 45 min at room temperature. The leaf discs were then washed gently three times with distilled water to remove unbound dye. Cells attached to the leaf surface were analysed macroscopically by the intensity of the blue dye.

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 OD₅₅₀ 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 % (v/v) paraformaldehyde and 1 % (w/v) tannic acid in 0.1 mol cacodylate buffer I⁻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://xccc.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 37 268 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. oryzae strains 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 K279a (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* pv. syringae 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 *Methylococcus 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* APECO1 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 NX₂LSX₂RAX₂VX₃L (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.

Fig. 1. Phylogenetic relationships between outer-membrane proteins MopB from xanthomonads and related genera. Abu, *Arcobacter butzleri*; Avi, *Azotobacter vinelandii*; Cfe, *Campylobacter fetus* subsp. *fetus*; Eco, *Escherichia coli*; Mca, *Methylococcus capsulatus*; Npr, *Nautilia profundicola*; Pae, *Pseudomonas aeruginosa*; Pfl, *Pseudomonas fluorescens*; Ppu, *Pseudomonas putida*; Psp, *Pseudomonas syringae* pv. *phaseolicola*; Pss, *Pseudomonas syringae* pv. *syringae*; Pst, *Pseudomonas syringae* pv. *tomato*; Sma, *Stenotrophomonas maltophilia*; Tde, *Thiobacillus denitrificans*; Xac, *Xanthomonas axonopodis* pv. *citri*; Xcc, *Xanthomonas campestris* pv. *campestris*; Xcv, *Xanthomonas campestris* pv. *vesicatoria*; Xoo, *Xanthomonas oryzae* pv. *oryzae*; Xf, *Xylella fastidiosa*.

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.

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.

(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 OD_{550} of about 3.7. The OD_{550} of Xc17 continued to increase slightly until saturation (OD_{550} 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 OD_{550} of the filtrates was measured. The results indicated that the OD_{550} 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. 3. Pathogenicity of Xcc strains. Cells (about 5×10^6 cells in 10 μ l) 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 OD_{550} 0.35 and grown at 28 °C. The OD_{550} 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 OD_{550} 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 μ M) 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) 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

Fig. 5. Aggregate formation and dispersal by Xcc strains. (a) Cells of Xc17, the *mopB* mutant XcMopB and the complemented strain XcMopB(pRK-*mopB*) were grown in LB at 28 °C for 16 h and treated by addition of DSF (final concentration, 10 μ M), 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.

Fig. 6. Growth of *Xcc* strains under stressful conditions. Overnight cultures of Xc17 and XcMopB were diluted into 30 ml LB medium to OD₅₅₀ 0.35. (a) Cells were grown at 28 or 37 °C to test for tolerance to elevated temperature. Growth of cells was monitored by reading of OD₅₅₀. (b) The pH of the LB medium was adjusted by addition of citrate buffer and growth was measured 16 h later. (c) SDS at different concentrations was added to the medium and growth of cells was measured 8 h later.

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 $\mu\text{g EPS ml}^{-1}$, approximately 58 % of that produced by wild-type Xc17 (3242 ± 203 $\mu\text{g ml}^{-1}$). The complemented strain, XcMopB(pRK-*mopB*), produced 2849 ± 256 $\mu\text{g EPS ml}^{-1}$, 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, mannan 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

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

Fig. 7. Electron microscopy of Xcc strains. Cells of the wild-type Xc17 (a), the *mopB* mutant XcMopB (c) and the complemented strain XcMopB(pRK-*mopB*) (e) were treated as described in Methods. (b), (d) and (f) are enlargements from (a), (c) and (e), respectively. Bars, 100 nm.

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 *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-*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-*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 *mopB* gene *in trans*. Previous SDS-PAGE analysis of OMPs from *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 *et al.*, 1993). These findings are consistent with our bioinformatic analysis showing that MopB is highly conserved among members of *Xanthomonas*. The Xcc MopB also has high identity with MopBs from members of the related genera *Stenotrophomonas* (>86.7 %) and *Xylella* (>68.4 %), but showed a low level of identity with OprF of *P. aeruginosa* (30.3 %) and OmpA of *E. coli* (20.8 %).

Mutation in the Xcc *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 *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 *Geobacter sulfurreducens*; marked changes in the amounts of several proteins, including a membrane-associated cytochrome, were observed (Afkar *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 *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

Fig. 8. Adhesion test of Xcc strains. (a) Overnight culture (100 μ l 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.

XccMopB, 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 (GumDMH1), 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

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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REFERENCES

- Afkar, E., Reguera, G., Schiffer, M. & Lovley, D. R. (2005). A novel *Geobacteraceae*-specific outer membrane protein J (OmpJ) is essential for electron transport to Fe(III) and Mn(IV) oxides in *Geobacter sulfurreducens*. *BMC Microbiol* **5**, 41.
- Barabote, R. D., Johnson, O. L., Zetina, E., San Francisco, S. K., Fralick, J. A. & San Francisco, M. J. (2003). *Erwinia chrysanthemi* *tolC* is involved in resistance to antimicrobial plant chemicals and is essential for phytopathogenesis. *J Bacteriol* **185**, 5772–5778.
- Becker, A., Katzen, F., Puhler, A. & Ielpi, L. (1998). Xanthan gum biosynthesis and application: a biochemical/genetic perspective. *Appl Microbiol Biotechnol* **50**, 145–152.
- Bishop, R. E. (2008). Structural biology of membrane-intrinsic beta-barrel enzymes: sentinels of the bacterial outer membrane. *Biochim Biophys Acta* **1778**, 1881–1896.
- Bourgault, R. & Bewley, J. D. (2002). Gel diffusion assays for endo-beta-mannanase and pectin methylesterase can underestimate enzyme activity due to proteolytic degradation: a remedy. *Anal Biochem* **300**, 87–93.
- Browning, D. F., Whitworth, D. E. & Hodgson, D. A. (2003). Light-induced carotenogenesis in *Myxococcus xanthus*: functional characterization of the ECF sigma factor CarQ and antisigma factor CarR. *Mol Microbiol* **48**, 237–251.
- Chan, J. W. & Goodwin, P. H. (1999). The molecular genetics of virulence of *Xanthomonas campestris*. *Biotechnol Adv* **17**, 489–508.
- Chao, N. X., Wei, K., Chen, Q., Meng, Q. L., Tang, D. J., He, Y. Q., Lu, G. T., Jiang, B. L., Liang, X. X. & other authors (2008). The *rsmA*-like gene *rsmA*(Xcc) of *Xanthomonas campestris* pv. *campestris* is involved in the control of various cellular processes, including pathogenesis. *Mol Plant Microbe Interact* **21**, 411–423.
- Choi, C. H., Lee, J. S., Lee, Y. C., Park, T. I. & Lee, J. C. (2008). *Acinetobacter baumannii* invades epithelial cells and outer membrane protein A mediates interactions with epithelial cells. *BMC Microbiol* **8**, 216.
- Chou, F. L., Chou, H. C., Lin, Y. S., Yang, B. Y., Lin, N. T., Weng, S. F. & Tseng, Y. H. (1997). The *Xanthomonas campestris* *gumD* gene required for synthesis of xanthan gum is involved in normal pigmentation and virulence in causing black rot. *Biochem Biophys Res Commun* **233**, 265–269.
- de Mot, R. & Vanderleyden, J. (1994a). The C-terminal sequence conservation between OmpA-related outer membrane proteins and MotB suggests a common function in both gram-positive and gram-negative bacteria, possibly in the interaction of these domains with peptidoglycan. *Mol Microbiol* **12**, 333–334.
- de Mot, R. & Vanderleyden, J. (1994b). A conserved surface-exposed domain in major outer membrane proteins of pathogenic *Pseudomonas* and *Branhamella* species shares sequence homology with the calcium-binding repeats of the eukaryotic extracellular matrix protein thrombospondin. *Mol Microbiol* **13**, 379–380.
- Dharmapuri, S. & Sonti, R. V. (1999). A transposon insertion in the *gumG* homologue of *Xanthomonas oryzae* pv. *oryzae* causes loss of extracellular polysaccharide production and virulence. *FEMS Microbiol Lett* **179**, 53–59.
- Dow, J. M., Crossman, L., Findlay, K., He, Y. Q., Feng, J. X. & Tang, J. L. (2003). Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc Natl Acad Sci U S A* **100**, 10995–11000.
- Dums, F., Dow, J. M. & Daniels, M. J. (1991). Structural characterization of protein secretion genes of the bacterial phytopathogen *Xanthomonas campestris* pathovar *campestris*: relatedness to secretion systems of other gram-negative bacteria. *Mol Gen Genet* **229**, 357–364.
- Dye, D. W., Bradbury, J. F., Goto, M., Hayward, A. C., Lelliott, R. A. & Schroth, M. N. (1980). International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. *Rev Plant Pathol* **59**, 153–168.
- Fu, J. F. & Tseng, Y. H. (1990). Construction of lactose-utilizing *Xanthomonas campestris* and production of xanthan gum from whey. *Appl Environ Microbiol* **56**, 919–923.
- Gotoh, N., Wakebe, H., Yoshihara, E., Nakae, T. & Nishino, T. (1989). Role of protein F in maintaining structural integrity of the *Pseudomonas aeruginosa* outer membrane. *J Bacteriol* **171**, 983–990.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- He, Y. W., Xu, M., Lin, K., Ng, Y. J., Wen, C. M., Wang, L. H., Liu, Z. D., Zhang, H. B., Dong, Y. H. & other authors (2006). Genome scale analysis of diffusible signal factor regulon in *Xanthomonas campestris* pv. *campestris*: identification of novel cell-cell communication-dependent genes and functions. *Mol Microbiol* **59**, 610–622.
- He, Y. W., Ng, A. Y., Xu, M., Lin, K., Wang, L. H., Dong, Y. H. & Zhang, L. H. (2007). *Xanthomonas campestris* cell-cell communication

- involves a putative nucleotide receptor protein Clp and a hierarchical signalling network. *Mol Microbiol* **64**, 281–292.
- Hsiao, Y. M., Fang, M. C., Sun, P. F. & Tseng, Y. H. (2009). Clp and RpfF up-regulate transcription of *pelA1* gene encoding the major pectate lyase in *Xanthomonas campestris* pv. *campestris*. *J Agric Food Chem* **57**, 6207–6215.
- Hsiao, Y. M., Liu, Y. F., Fang, M. C. & Tseng, Y. H. (2010). Transcriptional regulation and molecular characterization of the *manA* gene encoding the biofilm dispersing enzyme mannan endo-1,4-beta-mannosidase in *Xanthomonas campestris*. *J Agric Food Chem* **58**, 1653–1663.
- Jackson, D. W., Suzuki, K., Oakford, L., Simecka, J. W., Hart, M. E. & Romeo, T. (2002). Biofilm formation and dispersal under the influence of the global regulator CsrA of *Escherichia coli*. *J Bacteriol* **184**, 290–301.
- Katzen, F., Ferreira, D. U., Oddo, C. G., Ielmini, M. V., Becker, A., Puhler, A. & Ielpi, L. (1998). *Xanthomonas campestris* pv. *campestris* gum mutants: effects on xanthan biosynthesis and plant virulence. *J Bacteriol* **180**, 1607–1617.
- Keen, N. T., Tamaki, S., Kobayashi, D. & Trollinger, D. (1988). Improved broad-host-range plasmids for DNA cloning in gram-negative bacteria. *Gene* **70**, 191–197.
- Khan, N. A., Shin, S., Chung, J. W., Kim, K. J., Elliott, S., Wang, Y. & Kim, K. S. (2003). Outer membrane protein A and cytotoxic necrotizing factor-1 use diverse signaling mechanisms for *Escherichia coli* K1 invasion of human brain microvascular endothelial cells. *Microb Pathog* **35**, 35–42.
- Koebnik, R., Locher, K. P. & Van Gelder, P. (2000). Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* **37**, 239–253.
- Kostakioti, M., Newman, C. L., Thanassi, D. G. & Stathopoulos, C. (2005). Mechanisms of protein export across the bacterial outer membrane. *J Bacteriol* **187**, 4306–4314.
- Lee, M. C., Weng, S. F. & Tseng, Y. H. (2003). Flagellin gene *fliC* of *Xanthomonas campestris* is upregulated by transcription factor Clp. *Biochem Biophys Res Commun* **307**, 647–652.
- Lin, H. M. & Tseng, Y. H. (1979). Exopolysaccharide synthesis in *Xanthomonas oryzae*. *Proc Natl Sci Counc Repub China* **3**, 279–284.
- Lin, H. M., Tseng, H. C., Wang, C. J., Chyau, C. C., Liao, K. K., Peng, P. L. & Chou, F. P. (2007). Induction of autophagy and apoptosis by the extract of *Solanum nigrum* Linn in HepG2 cells. *J Agric Food Chem* **55**, 3620–3628.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Miller, V. L., Farmer, J. J., III, Hill, W. E. & Falkow, S. (1989). The *ail* locus is found uniquely in *Yersinia enterocolitica* serotypes commonly associated with disease. *Infect Immun* **57**, 121–131.
- Miller, V. L., Bliska, J. B. & Falkow, S. (1990). Nucleotide sequence of the *Yersinia enterocolitica* *ail* gene and characterization of the Ail protein product. *J Bacteriol* **172**, 1062–1069.
- Miller, V. L., Beer, K. B., Loomis, W. P., Olson, J. A. & Miller, S. I. (1992). An unusual *pagC*: *TnpA* mutation leads to an invasion- and virulence-defective phenotype in salmonellae. *Infect Immun* **60**, 3763–3770.
- Moore, J., Bailey, S. E., Benmechernene, Z., Tzitzilonis, C., Griffiths, N. J., Virji, M. & Derrick, J. P. (2005). Recognition of saccharides by the OpcA, OpaD, and OpaB outer membrane proteins from *Neisseria meningitidis*. *J Biol Chem* **280**, 31489–31497.
- Ojanen, T., Helander, I. M., Haahtela, K., Korhonen, T. K. & Laakso, T. (1993). Outer membrane proteins and lipopolysaccharides in pathovars of *Xanthomonas campestris*. *Appl Environ Microbiol* **59**, 4143–4151.
- Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Pal, U., Yang, X., Chen, M., Bockenstedt, L. K., Anderson, J. F., Flavell, R. A., Norgard, M. V. & Fikrig, E. (2004). OspC facilitates *Borrelia burgdorferi* invasion of *Ixodes scapularis* salivary glands. *J Clin Invest* **113**, 220–230.
- Prasadarao, N. V., Wass, C. A., Weiser, J. N., Stins, M. F., Huang, S. H. & Kim, K. S. (1996). Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infect Immun* **64**, 146–153.
- Rawling, E. G., Brinkman, F. S. & Hancock, R. E. (1998). Roles of the carboxy-terminal half of *Pseudomonas aeruginosa* major outer membrane protein OprF in cell shape, growth in low-osmolarity medium, and peptidoglycan association. *J Bacteriol* **180**, 3556–3562.
- Ray, S. K., Rajeshwari, R., Sharma, Y. & Sonti, R. V. (2002). A high-molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. *oryzae* exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence. *Mol Microbiol* **46**, 637–647.
- Rigano, L. A., Siciliano, F., Enrique, R., Sendin, L., Filippone, P., Torres, P. S., Questa, J., Dow, J. M., Castagnaro, A. P. & other authors (2007). Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv. *citri*. *Mol Plant Microbe Interact* **20**, 1222–1230.
- Rigden, D. J. & Galperin, M. Y. (2004). The DxDxDG motif for calcium binding: multiple structural contexts and implications for evolution. *J Mol Biol* **343**, 971–984.
- Rojas, C. M., Ham, J. H., Deng, W. L., Doyle, J. J. & Collmer, A. (2002). HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana glauca* seedlings. *Proc Natl Acad Sci U S A* **99**, 13142–13147.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sidhu, V. K., Vorholter, F. J., Niehaus, K. & Watt, S. A. (2008). Analysis of outer membrane vesicle associated proteins isolated from the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*. *BMC Microbiol* **8**, 87.
- Slater, H., Alvarez-Morales, A., Barber, C. E., Daniels, M. J. & Dow, J. M. (2000). A two-component system involving an HD-GYP domain protein links cell-cell signalling to pathogenicity gene expression in *Xanthomonas campestris*. *Mol Microbiol* **38**, 986–1003.
- Swings, J. G. & Civerolo, E. L. (1993). *Xanthomonas*. London: Chapman & Hall.
- Tadayyon, M., Gittins, J. R., Pratt, J. M. & Broome-Smith, J. K. (1994). Expression of membrane proteins in *Escherichia coli*. In *Membrane Protein Expression Systems: a User's Guide*, pp. 29–83. Edited by G. W. Gould. London: Portland Press.
- Tang, J. L., Liu, Y. N., Barber, C. E., Dow, J. M., Wootton, J. C. & Daniels, M. J. (1991). Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. *Mol Gen Genet* **226**, 409–417.
- Teng, C. H., Xie, Y., Shin, S., Di Cello, F., Paul-Satyaseela, M., Cai, M. & Kim, K. S. (2006). Effects of *ompA* deletion on expression of type 1 fimbriae in *Escherichia coli* K1 strain RS218 and on the association of

E. coli with human brain microvascular endothelial cells. *Infect Immun* **74**, 5609–5616.

Vieira, J. & Messing, J. (1991). New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* **100**, 189–194.

Vorholter, F. J., Schneiker, S., Goesmann, A., Krause, L., Bekel, T., Kaiser, O., Linke, B., Patschkowski, T., Ruckert, C. & other authors (2008). The genome of *Xanthomonas campestris* pv. *campestris* B100 and its use for the reconstruction of metabolic pathways involved in xanthan biosynthesis. *J Biotechnol* **134**, 33–45.

Wang, Y. (2002). The function of OmpA in *Escherichia coli*. *Biochem Biophys Res Commun* **292**, 396–401.

Wang, T. W. & Tseng, Y. H. (1992). Electrotransformation of *Xanthomonas campestris* by RF DNA of filamentous phage phi Lf. *Lett Appl Microbiol* **14**, 65–68.

Wengelnik, K., Marie, C., Russel, M. & Bonas, U. (1996). Expression and localization of HrpA1, a protein of *Xanthomonas campestris* pv.

vesicatoria essential for pathogenicity and induction of the hypersensitive reaction. *J Bacteriol* **178**, 1061–1069.

William, P. H. (1980). Black rot: a continuing threat to world crucifers. *Plant Dis* **64**, 736–742.

Woodruff, W. A. & Hancock, R. E. (1989). *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. *J Bacteriol* **171**, 3304–3309.

Yang, B. Y. & Tseng, Y. H. (1988). Production of exopolysaccharide and levels of protease and pectinase activity in pathogenic and non-pathogenic strains of *Xanthomonas campestris* pv. *campestris*. *Bot Bull Acad Sin* **29**, 93–99.

Yang, C. H., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., Chen, J. W., Perna, N. T. & Keen, N. T. (2002). *hrp* genes of *Erwinia chrysanthemi* 3937 are important virulence factors. *Mol Plant Microbe Interact* **15**, 472–480.

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