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Functional properties of the major outer membrane protein in *Stenotrophomonas maltophilia*

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Stenotrophomonas maltophilia **is an opportunistic pathogen that is closely associated with high morbidity and mortality in debilitated and immunocompromised individuals. Therefore, to investigate the pathogenesis mechanism is urgently required. However, there are very few studies to evaluate the functional properties of outer membrane protein, which may contribute to the pathogenesis in *S. maltophilia*. In this study, three abundant proteins in the outer membrane fraction of *S. maltophilia* were identified by liquid chromatography-tandem mass spectrometry as OmpW1, MopB, and a hypothetical protein. MopB, a member of the OmpA family, was firstly chosen for functional investigation in this study because many OmpA-family proteins are known to be involved in pathogenesis and offer potential as vaccines. Membrane fractionation analyses demonstrated that MopB was indeed the most abundant outer membrane protein (OMP) in *S. maltophilia*. For functional studies, the *mopB* mutant of *S. maltophilia* (SmMopB) was constructed by insertional mutation. MopB deficiency resulted in a change in the protein composition of OMPs and altered the architecture of the outer membrane. The SmMopB strain exhibited reduced cytotoxicity toward L929 fibroblasts and was more sensitive to numerous stresses, including human serum, sodium dodecyl sulfate, and hydrogen peroxide compared with wild-type *S. maltophilia*. These results suggest that MopB may be a good candidate for the design of vaccines or anti-MopB drugs for controlling serious nosocomial infections of multidrug-resistant *S. maltophilia*, especially in immunosuppressed patients.**

Keywords**:** outer membrane protein, *Stenotrophomonas maltophilia*, pathogenesis

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Introduction

Outer membrane protein A (OmpA) may act as an adhesin, invasin, or immune target, and plays a role in bacterial conjugation, bacteriophage binding, and maintenance of cellular structural integrity (Smith *et al.*, 2007). Several OmpA-family outer membrane proteins (OMPs), including OmpA of *Klebsiella pneumoniae* (Pichavant *et al.*, 2003), OmpA of *Escherichia coli* (Khan *et al.*, 2003), OmpA of *Acinetobacter baumannii* (Choi *et al.*, 2008), and OprF of *Pseudomonas aeruginosa* (Azghani *et al.*, 2002), have been shown to be involved in pathogenesis. The *ompA* mutant strains of these bacteria are much less invasive to host cells than their parental strains (Azghani *et al.*, 2002; Khan *et al.*, 2003; Choi *et al.*, 2008; Hsieh *et al.*, 2013). In addition, pretreatment of cells with exogenous purified OmpA or anti-OmpA antibody significantly reduces bacterial adherence to host cells (Azghani *et al.*, 2002; Choi *et al.*, 2008; Khan *et al.*, 2003). Thus, investigations of the role of OmpA-family OMPs in bacteria have provided novel insights into bacterial pathogenesis and infection.

The non-fermenting Gram-negative bacterium *Stenotrophomonas maltophilia*, originally given the name *Pseudomonas maltophilia* and later *Xanthomonas maltophilia*, is significant because of its nosocomial infections and the clinical syndrome it causes, which includes bacteremia, respiratory tract infection, endocarditis, urinary tract infection, meningitis, and wound infection, particularly in immunosuppressed patients (Denton and Kerr, 1998; Brooke, 2012). Many clinical isolates of *S. maltophilia* are highly resistant to antimicrobial agents, including aminoglycosides and β-lactam, and have become a therapeutic challenge (Brooke, 2012). In many bacterial pathogens, OMPs are recognized as antigens by host immune defenses and may play a role in pathogenesis (Jeannin *et al.*, 2002; Ellis and Kuehn, 2010). Currently, the OMPs of *S. maltophilia* are not well characterized, and little is known about the virulence-related factors and mechanisms involved in the pathogenesis of *S. maltophilia*.

In this study, we sought to characterize the major OMPs in *S. maltophilia* and investigate their functional roles. We demonstrated that MopB, a member of the OmpA family, is a major OMP in *S. maltophilia*. Mutation of the corresponding gene resulted in changes in cell surface structure, increased sensitivity to hydrogen peroxide, sodium dodecyl sulfate (SDS) and human serum, and reduced cytotoxicity and adhesion ability. We suggest that MopB may be a possible candidate for the design of vaccines or drugs for controlling *S. maltophilia* infections.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strain, plasmids, and primers used in this study are listed in Table 1. Bacterial strains were grown in Luria-Bertani (LB) broth or LB agar (Miller, 1972) at 30°C (*S. maltophilia* strains) or 37°C (*E. coli*). For measurements of cell growth, overnight cultures were diluted into 30 ml LB medium to obtain an initial OD_{600} value of 0.1, followed by incubation at 30°C and measurement of OD_{600} values at appropriate intervals. Sensitivity to hydrogen peroxide (up to 0.8% w/v) and SDS (up to 0.02% w/v) was investigated by adding the chemical agent and measuring bacterial growth by monitoring OD_{600} values. Unless otherwise indicated, the antibiotics ampicillin, kanamycin, tetracycline, and gentamicin were added at final concentrations 50, 50, 15, and 15 mg/ml, respectively, when required.

Enzymes and chemicals

Restriction endonucleases were purchased form TaKaRa Biomedicals. *Taq* DNA polymerase and polymerase chain reaction (PCR)-related materials were obtained from Merck or Takara Biochemicals. Laboratory-grade chemicals were purchased from Sigma Chemical Company or Merck.

Cloning and sequencing of the *mopB* gene

The *mopB* gene was cloned by first designing a set of primers (*mop*BS1 and *mop*BS2) based on the genomic sequence of *S. maltophilia* K279a. PCR amplification was performed using *S. maltophilia* ATCC13637 chromosomal DNA as a template. PCR products containing the putative promoter region and *mopB* gene were cloned into the yT&A vector (Yeastern). Both strands of the cloned DNA were sequenced.

Insertional inactivation of the *mopB* gene

SmMopB, the *S. maltophilia mopB* mutant strain, was constructed by insertional mutation. A partial DNA fragment of *mopB* was amplified by PCR with primers mopB-F and mopB-R using *S. maltophilia* ATCC13737 chromosomal DNA as a template, and then ligated into yT&A vector (Yeastern Biotech,) to yield yT&A-*mopB*. After sequence verification, the partial fragment of *mopB* was excised from this construct using *Kpn*I and *Bgl*II, and ligated into pOK12-Tc to create pOKTc-*mopB* (Fig. 1A). The resulting plasmid pOKTc-*mopB* was introduced into strain *S. maltophilia* by electroporation. *S. maltophilia* ATCC13637 is highly resistant to several antibiotics (ampicillin, kanamycin, tetracycline, gentamicin). Accordingly, the mutant strain was selected by an alternative method, using LB medium supplemented with tetracycline at a concentration of 60 mg/ml, a concentration slightly higher than the minimum inhibitory concentration (MIC) for *S. maltophilia* ATCC13637. The *mopB* mutant was confirmed by PCR using two set of primers: set 1, mopBAF and MCS-*Hin*dIII; set 2, mopBAR and MCS-*Spe*I (Fig. 1B).

Serum bactericidal activity assay

Serum bactericidal assays were performed as described previously (McKay *et al.*, 2003). Bacteria (2×10^9) were suspended in 500 µl sterile 0.85% (w/v) NaCl, after which an

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Reference or source
S. maltophilia		
S. maltophilia 13637	ATCC type strain, Ap^r, Tc^r, Gm^r, Km^r	Hugh and Ryschenkow (1961)
SmMopB	*mopB* mutant derived from Sm13637, Ap^r, Tc^r, Gm^r, Km^r	This study
X. campestris pv. campestris		
Xc17	Virulent wild-type strain isolated in Taiwan, Ap^r	Yang and Tseng (1988)
XcMopB	*mopB* mutant derived from Xc17, Ap^r, Km^r	Chen *et al.* (2010)
E. coli		
DH5α	*endA1 hsdR17*($rk^- mk^+$) *supE44 thi-1 recA1 gyrA relA1* 80d *lacZ* DM15D(lacZYA-argF) *U169*; general cloning host	Hanahan (1983)
Plasmid		
yT&A vector	PCR cloning vector, Ap^r	Yeastern
yT&A-*mopB*	A partial fragment amplified from *mopB* and cloned into yT&A vector, Ap^r, Tc^r	This study
pOK12-Tc	*E. coli* general cloning vector derived from P15A replicon, with *lacZ* fragment, Km^r, Tc^r	Lee *et al.* (2001)
pOKTc-*mopB*	A partial fragment amplified from *mopB* and cloned into pOK12-Tc, Km^r, Tc^r	This study
pFY-$mopB_{Sm}$	A DNA fragment contained full-length *mopB* and cloned into pFY13-9, Tc^r	This study
Primers		
mopBS1	5′-ACCCTTGCAGCGTGAGGGTCTT-3′	This study
mopBS2	5′-GCCGGGTCCTGCTGTGTCCTA-3′	This study
mopB-F	5′-GAAGGCCGCGGCTGGAACCC-3′	This study
mopB-R	5′-CCGGGTAACGCTTCAGGATCTCGG-3′	This study
mopBAF	5′-ATGAACAAGAAGATCCTTACTGCCGC-3′	This study
MCS-*Hin*dIII	5′-AAGCTTCGAATTCGAGCTCCCGG-3′	This study
mopBAR	5′-TTAGTTCTGGACGTTCAGCTCGGTACG-3′	This study
MCS-*Spe*I	5′-GGTGATCAGGCTCCGGAGCTCTAGA-3′	This study

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

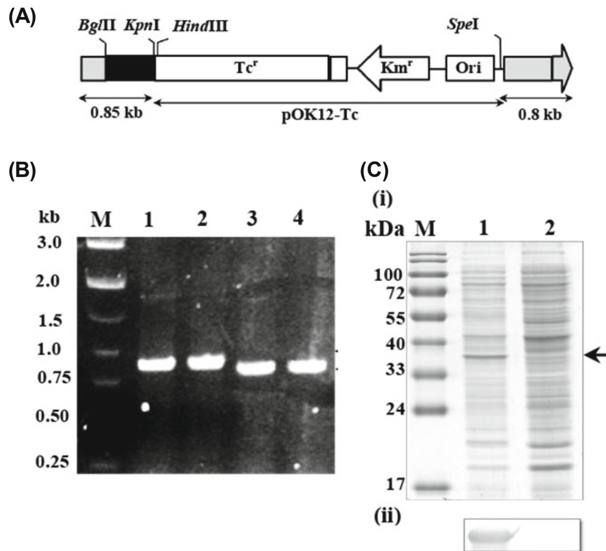

Fig. 1. Construction of the *S. maltophilia mopB* mutant. (A) Schematic representation of the *mopB* mutant. Construction of the *mopB* mutant by insertion of pOKTc-*mopB* into *S. maltophilia* through homologous recombination. Partial *mopB* carried by pOKTc-*mopB* is shown on a black background, whereas full-length *mopB* encoded by *S. maltophilia* 13637 is shown in gray. (B) Insertion was verified by PCR. Lanes: M, size markers; 1 and 2, 0.85 kb DNA fragment amplified by the primers MopBAF and MCS-*Hin*dIII; 3 and 4, 0.8 kb DNA fragment amplified by the primers MopBAR and MCS-*Spe*I. (C) Insertion was verified by Western blotting. The crude extract proteins prepared from *S. maltophilia* ATCC13637 and SmMopB were separated by SDS-PAGE on 12% gels (i), followed by Western blotting using polyclonal antibodies against $MopB_{Xcc}$ (ii). Lanes: M, size markers; 1, crude extract prepared from *S. maltophilia* ATCC13637; 2, crude extract prepared from SmMopB. Arrow indicates the position of MopB.

equal volume of adult normal human serum (NHS), obtained from eight healthy individuals, was added. The bacteria and serum mixture was then incubated at 37°C for 1 h. Aliquots (100 µl) were then removed, diluted appropriately, and plated on LB agar plates. Bacteria numbers were determined by counting colonies. In some experiments, NHS was incubated with 1 mM EGTA for 20 min or heat-inactivated at 56°C for 30 min prior to use.

Fractionation of *S. maltophilia* cells

The procedures used for fractionation of *S. maltophilia* were as described previously (Chen *et al.*, 2010). Protein concentrations in Sarkosyl-soluble and Sarkosyl-insoluble fractions were determined using a Bio-Rad detergent-compatible protein assay kit (catalog no. 500-0012) and a regular Bio-Rad protein assay kit (catalog no. 500-0006), respectively.

Liquid chromatography-tandem mass spectrometry

The outer membrane proteins from *S. maltophilia* were mixed with sample buffer, heated in a boiling water bath for 5 min, and following subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation in 12% (w/v) polyacrylamide gel. Following proteins were visualized by staining the gels with Coomassie brilliant blue, excised from the gels, and subjected to liquid chromatography-tandem mass spectrometry (LCMS/MS) (ABI Qstar System) analysis at the Biotechnology Center, National Chung Hsing University as described previously (Lee *et al.*, 2009).

Cytotoxicity assay

L929 fibroblasts (1×10^4), cultured in Dulbecco's Modified Eagle Medium containing 10% fetal calf serum (Hyclone Laboratories), were plated in 96-well plates and incubated at 37°C in a humidified 5% CO_2 atmosphere. Bacterial toxicity was determined by adding 2×10^7 bacteria to L929 fibroblast cells and measuring lactate dehydrogenase (LDH) activity in the medium (reflecting LDH released by dead/dying cells) using an LDH cytotoxicity detection kit.

Adhesion test

The adhesion of *S. maltophilia* strains was quantitatively assessed as described previously (Jackson *et al.*, 2002; Chao, *et al.*, 2008). Cells from overnight cultures (100 µl aliquots, OD_{600} = 1.0) were inoculated into 3.0 ml of LB medium in 20-ml universal tubes (QingFa) and incubated with shaking

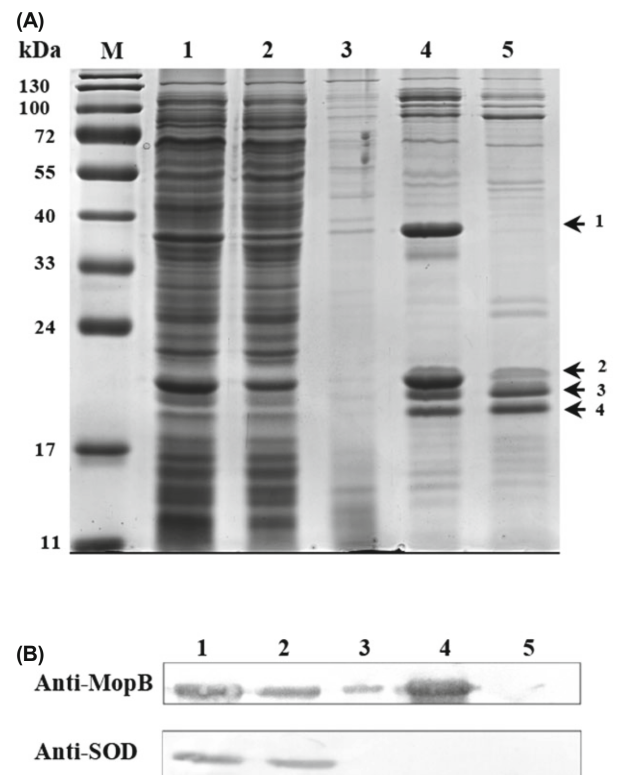

Fig. 2. SDS-PAGE analysis of the OMP profiles of *S. maltophilia*. (A) Total membrane proteins prepared from *S. maltophilia* ATCC13637 and SmMopB were treated with 0.25% Sarkosyl and separated into detergent-soluble and -insoluble fractions. Each fraction was separated by SDS-PAGE on 12% gels, followed by Western blotting using polyclonal antibodies against against $MopB_{Xcc}$ and SOD_{Xcc} (B). Lanes: M, size markers; 1, crude extract prepared from *S. maltophilia* ATCC13637; 2, proteins from cytoplasm and periplasm prepared from *S. maltophilia* ATCC13637; 3, Sarkosyl-soluble fraction prepared from *S. maltophilia* ATCC13637; 4, Sarkosyl-insoluble fraction prepared from *S. maltophilia* ATCC13637; 5, Sarkosyl-insoluble fraction prepared from SmMopB. Arrows indicate proteins further identified using LC-MS/MS, as shown in Table 2.

at 30°C for 6 h. The cells that bound to the glass surface were measured by removing the medium, rinsing the tubes with 3 ml of distilled water (three times), and staining with 2 ml of 0.3% (w/v) crystal violet (Bio Star). Crystal violet associated with bound cells was solubilized in 33% (v/v) acetic acid and measured at OD_{630} using a Hitachi U-1900 spectrophotometer.

Electron microscopy

Bacteria incubated in LB were washed with phosphate-buffered saline, passed through 0.22-μm membrane (Millipore), and then fixed for 4 h in 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate. Filter membranes were immersed in 1% (w/v) osmium tetroxide for 1 h and then dehydrated with an ethanol gradient (50%, 70%, 90%, and 100%), immersing membranes twice in each solution for 10 min, and then immersed in acetone. Membranes were dried in a Tousimis PVT-3B critical point dryer. The specimens were then sputter coated with a JFC-1100E ion-sputtering device and viewed with a JSM-7401F scanning electron microscope (JEOL).

Results and Discussion

Identification of the abundant OMPs in *S. maltophilia*

To profile the OMPs of *S. maltophilia*, we fractionated *S. maltophilia* cells, separating membrane proteins into soluble inner membrane proteins and insoluble OMPs based on differential solubility in Sarkosyl (0.25%, w/v). Proteins in each fraction were then separated by SDS-PAGE on 12% polyacrylamide gels (Fig. 2A). Antibodies against superoxide dismutase (SOD_{Xcc}), a protein present only in the cytosol, were used as a control for sample quality. The results showed that SOD protein was detected in crude extracts and in fraction containing cytoplasmic and periplasmic proteins (Fig. 2B).

As shown in Fig. 2A, the four most abundant OMP bands detected in Coomassie Brilliant Blue-stained gels were selected and further analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). These proteins corresponded to three distinct proteins (Table 2). Proteins 1 and 2 were both annotated as OmpA/MotB domain OMP. The molecular weight of protein 1 was about 37 kDa, similar to that of the major OMP in *X. campestris* strains (Ojanen *et al.*, 1993). Protein 2 may be a degraded form of protein 1 that arises through unknown cellular processing events. Protein 3 was identified as OmpW1, and protein 4 was identified as a hypothetical protein without any conserved domains.

Numerous studies have reported a multitude of functions for OmpA-related proteins, establishing their importance in bacterial pathogenesis and vaccine development (Smith *et al.*, 2007; Maiti *et al.*, 2011). OmpW, a member of a family of small outer membrane β-barrel proteins, is an approximately 200-amino-acid (aa) protein that is widespread among Gram-negative bacteria (Saier *et al.*, 2006). OmpW is also found in all known strain of *Vibrio cholera*, and has attracted interest for vaccine development (Das *et al.*, 1998). The roles of the OmpA/MotB-domain protein and OmpW1 in *S. maltophilia* have not been explored. However, a bioinformatic analysis has annotated the OmpA/MotB-domain protein in *X. campestris* pv. campestris strain 33913 as $MopB_{Xcc}$. Thus, the OmpA/MotB-domain protein in *S. maltophilia*, which was the most abundant OMP, was named $MopB_{Sm}$ and was chosen as the primary target for further investigation.

Characterization of MopB in *S. maltophilia*

The MopB protein of *S. maltophilia* ATCC13637, deduced from the gene (1,092 bp; HQ399464), is a 363-aa protein with an N-terminal signal peptide of 22 aa and a conserved C-terminal OmpA domain. Removal of the signal peptide is predicted to produce a mature protein of 36,844 Da with a pI of 4.89.

A comparative analysis revealed that MopB is highly conserved among *Stenotrophomonas*. In sequence alignments, *S. maltophilia* ATCC13637 MopB had a high level of identity with homologs from *S. maltophilia* strains R551-3 (91.5%; YP_002027188) and K279a (88.3%; YP_001970833), and *Stenotrophomonas* sp. SKA14 (90.9%; ZP_05136974). *S. maltophilia* ATCC13637 MopB also had a high level of identity to MopB from other evolutionarily closely related members of *Xanthomonas*, including *X. axonopodis* pv. citri str. 306 (84.7%; NP_641359); *X. campestris* pv. vesicatoria str. 85–10 (84.7%; YP_362773); *X. oryzae* pv. oryzicola strain BLS256 (84.4%; ZP_02242092); *X. oryzae* pv. oryzae strains PX099A (84.1%; YP_001915346) and MAFF 311018 (84.1%; BAE-70245); three strains of *X. campestris* pv. campestris (83.8%; NP_636321, YP_244364, and YP_001904823); and four strains of *Xylella fastidiosa* (64.1%-66.7%; NP_779898, NP_297633, YP_001830479, and YP_001776390). *S. maltophilia* ATCC-13637 MopB shared a low degree of identity with MopB from *Methylococcus capsulatus* str. Bath (44.4%; YP_115490), OprF from *P. aeruginosa* PA01 (31.0%; NP_250468), and OmpA from *E. coli* (21.7%; YP_852080).

MopB deficiency causes changes in the protein composition of the outer membrane

To further study the function of MopB, we constructed a *mopB* mutant strain (SmMopB), as described in 'Materials and Methods'. This mutant and the parental strains were used for comparison of OMP profiles and to verify that the

Table 2. OMPs of *S. maltophilia* identified by LC-MS/MS

Protein number	Accession number	Protein name	Calculated mass (M_r)	Calculated pI	Sequence coverage
1	YP_002027188	OmpA/MotB domain protein (*S. maltophilia* R551-3)	38,920	4.80	57%
2	YP_002027188	OmpA/MotB domain protein (*S. maltophilia* R551-3)	38,920	4.80	24%
3	ZP_05136211	OmpW1 (*S.* sp. SKA14)	22,709	8.75	39%
4	YP_002026659	hypothetical protein (*S. maltophilia* R551-3)	21,071	5.80	50%

Fig. 3. **MopB deficiency causes changes in cell surface structure.** Cells of the wild-type strain of *S. maltophilia* (Sm ATCC13637) (A) and SmMopB (B) were observed by scanning electron microscopy. (C) and (D) are enlargements from (A) and (B), respectively.

most abundant protein band was indeed MopB. In the outer membrane fraction, the most abundant protein bands identified as MopB (protein 1 and 2) in the wild-type strain were lost in SmMopB (Fig. 2A; lane 4 and 5). In addition, SDS-PAGE analyses showed significant differences in protein composition between wild-type *S. maltophilia* and the SmMopB mutant strain; in the latter, some previously identified proteins were absent and some new proteins were present (Fig. 2A; lanes 4 and 5). Taken together, these results indicate that MopB of *S. maltophilia* is indeed the most abundant OMP, and further demonstrate that a MopB deficiency results in a marked alteration in the protein composition of the outer membrane.

Kustos *et al.* (2007) suggested that a change in OMP composition might alter antibiotic sensitivity and pathogenicity. Thus, we tested parental and *mopB* mutant strains for sensitivity to the antibiotics ampicillin, cefoxitin, cefotaxime, chloramphenicol, oxacillin, and piperacillin. No significant differences were found between the parental and SmMopB strains (data not shown). In addition to its structural properties, OmpA serves as a receptor for many bacteriophages (Morona *et al.*, 1984). We thus infected parental and SmMopB strains with several bacteriophages isolated from *S. maltophilia*, and found that mutation of the *mopB* gene had no effect on the normal bacteriophage infection process (data not shown). Thus, the roles of proteins uniquely present or absent in the outer membrane of the *mopB* mutant remain elusive. Further studies will be needed to identify these proteins and establish the significance of their differential expression.

MopB deficiency causes changes in cell surface structure

Major OMPs have been shown to play a role in the structural integrity of the bacterial cell surface (Sonntag *et al.*, 1978; Koebnik *et al.*, 2000; Smith *et al.*, 2007). To test whether mutation of the major OMP MopB altered the architecture of the outer membrane, we examined *S. maltophilia* cells

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S.maltophilia     NFDFDKSNLRPDAVAILSEATEILKRYP--DLRVEVAGHTDS-KGTDAYNQKLSERRATA
E.coli            LFNFNKATLKPEGQAALDQLYSQLSNLDPKDGSVVVLGYTDR-IGSDAYNQGLSERRAQS
K.peuemoniae      LFNFNKATLKPEGQQALDQLYTQLSNMDPKDGSAVVLGYTDR-IGSEAYNQQLSEKRAQS
N.meningitidis    LFGFDKDSLRAEAQDNLKVLAQRLGQTN--IQSVRVEGHTDF-MGSDKYNQALSERRAYV
PG33              YFRINSAKIDRNQEINVYNTAEYAKTNN---APIKVVGYADEKTGTAAYNMKLSERRAKA
PG32              LFRFDSHVVDKDQLINLYDVAQFVKETN---EPITVVGYADP-TGNTQYNEKLSERRAKA

S.maltophilia     VYNYLTKNGVDAGRLVGPIGYGESRPIAPNTNPD-------------GSDNPEGRAKNR
E.coli            VVDYLISKGIPADKIS-ARGMGESNPVTGNTCDN-----------VKQRAALIDCLAPDR
K.peuemoniae      VVDYLVAKGIPAGKIS-ARGMGESNPVTGNTCDN-----------VKARAALIDCLAPDR
N.meningitidis    VANNLVSNGVPVSRIS-AVGLGESQAQMTQVCEAEVAKLGAKVSKAKKREALIACIEPDR
PG33              VAKMLE-KYGVSADRITIEWKGSSEQIYEENAWN-------------------------R
PG32              VVDVLTGKYGVPSELISVEWKGDSTQPFSKKAWN-------------------------R

S.maltophilia     RTELNVQN--------------- 378
E.coli            RVEIEVKGIKDVVTQPQA----- 351
K.peuemoniae      RVEIEVKGYKEVVTQPAA----- 361
N.meningitidis    RVDVKIRSIVTRQVVPAHNHHQH 242
PG33              IVVMTAAE--------------- 395
PG32              VVIVRSK---------------- 407
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Fig. 4. **Multiple sequence alignment of C-terminal amino acids of *S. maltophilia* MopB and five verified OmpAs.** The amino acid sequence of *S. maltophilia* MopB was aligned with five experimentally verified, immunogenic OmpAs. The experimentally verified OmpAs used for the alignment were *E. coli* OmpA (NP_415477.1), *K. pneumoniae* OmpA (ABR76422), *N. meningitidis* OmpA (YP_001599860), *P. gingivalis* PG33 OmpA (AF175715), and *P. gingivalis* PG32 OmpA (AF175714). Identical residues are shown on a black background, whereas conserved residues are shadowed. Asterisks indicate the residues reported to directly interact with peptidoglycans. Dots indicate the residues reported to indirectly interact with peptidoglycans.

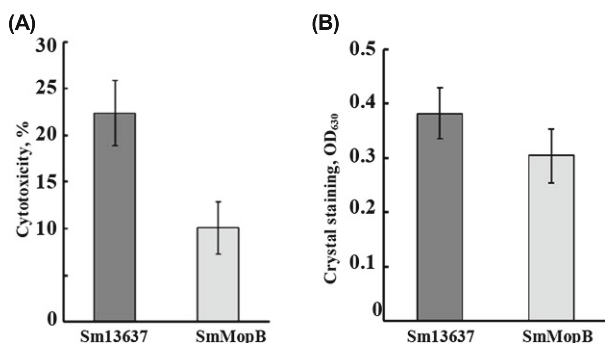

Fig. 5. Mutation in the *S. maltophilia mopB* gene causes reduced cytotoxicity and adhesion ability. (A) Cytotoxicity assays were performed by incubating bacterial cells with L929 fibroblasts and measuring the number of surviving cells after 6 h. (B) Adhesion assays were performed by incubating *S. maltophilia* strains in 20-ml Universal tubes (QingFa) with shaking at 30°C for 6 h and measuring crystal violet-stained cells that bound to the glass surface.

by electron microscopy. SmMopB had a roughness surface and more fibrin-like materials as compared with wild type strain (Fig. 3), suggesting that the changes in OMP composition associated with *mopB* mutation (Fig. 2A) may be responsible for deforming the mutant surface.

The conservation of the C-terminal OmpA domain between *S. maltophilia* MopB and experimentally verified OmpAs from *E. coli* (OmpA, NP_415477.1), *K. pneumoniae* (OmpA, ABR76422), *Neisseria meningitidis* (RmpM, YP_001599860), *Porphyromonas gingivalis* (PG33, AF175715), and *P. gingivalis* (PG32, AF175714) is depicted in Fig. 4, while their N-terminal sequences shared low similarities. The C-terminal domain of *N. meningitides* RmpM contains several conserved residues previously demonstrated to be involved in direct (D41, Y49, R57, and R120) and indirect (F2, G38, G45, N50, L53, and G82) interactions with peptidoglycans (Grizot and Buchanan, 2004). These conserved residues are also present in *S. maltophilia* MopB (Fig. 4), consistent with a similar peptidoglycan-interacting function for MopB and a possible role in the maintenance of membrane and cell morphology. Accordingly, we speculate that MopB-deficiency reduces the association between the outer membrane and peptidoglycan, and contributes to the rough shape observed in electron micrographs.

Mutation in the *mopB* gene causes a reduction in the cytotoxicity and adhesion ability of *S. maltophilia*

Many OmpA family proteins are known to be involved in bacterial pathogenesis and are capable of activating innate immunity and inducing cytotoxic responses (Khan *et al.*, 2003; Mortensen and Skaar, 2012; Pore *et al.*, 2012). To test the cytotoxic effect of MopB, we incubated wild-type and *mopB* mutant bacterial cells with L929 fibroblasts, and determined the number of dead fibroblast cells as described in 'Materials and Methods'. As shown in Fig. 5A, the cytotoxic effect of the parental strain (22.4%) was approximately 2-fold higher than that of the SmMopB strain (10%), indicating that inactivation of the *mopB* gene in *S. maltophilia* reduced cytotoxicity toward L929 fibroblasts.

Windhorst *et al.* (2002) have proposed that extracellular proteases play an important role in the pathogenesis of *S. maltophilia*. To address this, we assayed the hydrolytic activities of wild-type and *mopB* mutant cells on XOLN agar plates supplemented with 1% skim milk. We found no sig-

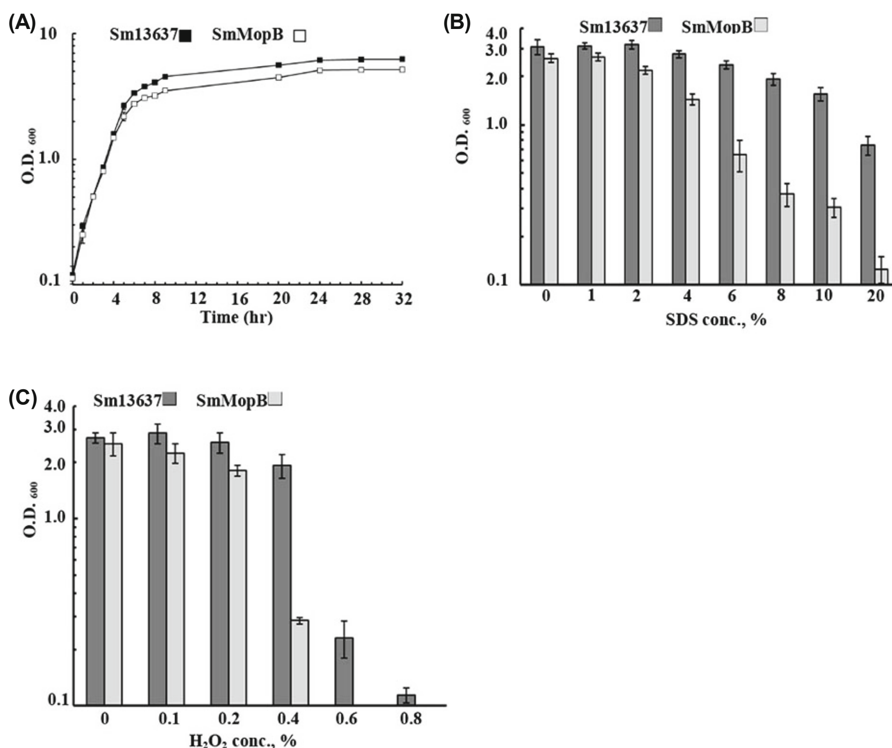

Fig. 6. Physiological analysis of SmMopB. Overnight cultures of *S. maltophilia* ATCC-13637 and SmMopB were diluted into 30 ml LB medium to an OD_{600} of 0.1. (A) Growth of *S. maltophilia* 13637 and SmMopB in LB was monitored by measuring OD_{600} at intervals. SDS (B) or hydrogen peroxide (C) at different concentrations was added to the medium and the growth of cells was measured 8 h later.

nificant differences between parental and SmMopB strains (data not shown). In addition, transwell assays demonstrated that extracellular (secreted) fractions from parental and *mopB* mutant strains of *S. maltophilia* had similar cytotoxic effects on L929 fibroblasts (data not shown). These results suggest that extracellular enzymes were not important virulence factors in the cytotoxic effect of *S. maltophilia*.

Adhesion, colonization, and biofilm formation by many human pathogens are important for their environmental persistence, spread, and opportunistic infection of hosts (Hall-Stoodley and Stoodley, 2005). We thus tested the abiotic adhesion ability of *S. maltophilia* strains using a crystal violet staining assay, as described in 'Materials and Methods'. As shown in Fig. 5B, the OD_{630} value of samples prepared from SmMopB cells (OD_{630} = 0.33) was 78.6% that of the wild type strain (OD_{630} = 0.42), suggesting that MopB-deficiency reduced the ability of *S. maltophilia* to adhere to an abiotic surface. Accordingly, we speculated that the infectivity of MopB-deficient *S. maltophilia* strains might be reduced.

SmMopB is more sensitive to stressful conditions than the parental strain

Many OMP mutant strains are significantly more sensitive to detergents and environmental stresses than their corresponding wild-type strains (Wang, 2002). Therefore, we evaluated the effects of mutation of *mopB* on *S. maltophilia* sensitivity to various stresses.

SmMopB grows more slowly than the parental strain: The growth rate of SmMopB was similar to that of the parental strain before entering the stationary phase (Fig. 6A). However, at 8 and 24 h, the OD_{600} value of the SmMopB culture was reduced slightly to about 78% (3.2/4.1) and 82% (5.1/6.2) that of the parental strain, respectively (Fig. 6A).

In *X. campestris* pv. campestris, in which the *mopB* mutant (XcMopB) and parental strains also grow at similar rates prior to the stationary phase, the *mopB* mutant strain forms obvious aggregates upon entering the stationary phase (Chen *et al*., 2010). In contrast to *X. campestris mopB* mutant, MopB-deficient *S. maltophilia* did not form aggregates in LB or XOLN broth (data not shown); thus, despite the fact that $MopB_{xcc}$ and $MopB_{sm}$ are approximately 84% identical at the amino acid sequence level, the corresponding *X. campestris* and *S. maltophilia mopB* mutant strains exhibited distinctive aggregation behavior. Interestingly XcMopB (pFY-$mopB_{Sm}$) can reverse *X. campestris* pv. campestris *mopB* mutant from aggregated state to dispersed state (data not shown), indicating that $MopB_{Sm}$ and $MopB_{Xcc}$ shared similar structure and function.

SmMopB is more sensitive to SDS and hydrogen peroxide: To test for effects of environment stresses on the SmMopB strain, we added different concentrations of SDS or hydrogen peroxide to bacterial cultures. The tolerance of the SmMopB strain to SDS or hydrogen peroxide decreased markedly with increasing concentrations of SDS or hydrogen peroxide (Fig. 6B and C). As shown in Fig. 6B, the wild-type strain was able to tolerate SDS at a concentration of 0.02% (w/v); in contrast, at this concentration, the total yield of SmMopB cells was reduced to about 4.7% of that in the absence of SDS. As shown in Fig. 6C, there was no detectable growth of *mopB* mutant cells exposed to a hydrogen peroxide concentration of 0.6% (w/v), whereas the growth of the wild-type strain at this concentration was about 8.1% of that observed at a concentration of 0.1%.

MopB increases *S. maltophilia* resistance to complement-mediated bactericidal activity: Serum resistance is a phenomenon frequently exhibited by Gram-negative pathogens associated with systemic infections, especially in bacteremia and septicemia (Nishio *et al*., 2005). The *E. coli* K1 strain *ompA* mutant is more sensitive to the bactericidal effect of pooled human serum than the wild-type strain (Weiser and Gotschlich, 1991). Thus, to test whether the *mopB* gene is also an important determinant of resistance to serum in *S. maltophilia*, we assessed serum-mediated bactericidal activity. As shown in Fig. 7, 53.3% of *S. maltophilia* ATCC13637 survived in NHS, whereas only 11.7% of SmMopB survived, suggesting that MopB has a protective effect against the serum-mediated killing reaction in *S. maltophilia*. In addition, incubation of parental or SmMopB strains with heat-inactivated NHS (to denature complement activity) restored serum resistance (Fig. 7), indicating that complement is important for the serum-mediated killing of *S. maltophilia* cells. To determine which complement pathway was responsible for NHS-mediated killing of *S. maltophilia*, we incubated *S. maltophilia* ATCC13637 and SmMopB with NHS supplemented with 1 mM EGTA. NHS supplemented with EGTA did not show bactericidal activity in either strain (Fig. 7), indicating that the classical complement pathway might be responsible for the killing activity of serum and suggesting a role for MopB in resistance to complement-mediated bactericidal activity in *S. maltophilia*. In conclusion, mutation of major OMP MopB were accompanied by altering composition of OMP profiles and deformation of mutant surface, as revealed by fractionation of OMP and electron microscopy. Therefore, the marked change in the OMP profiles and surface structure observed in SmMopB maybe the reason for the multiple defects including reduced cytotoxicity, and more sensitive to environmental stresses.

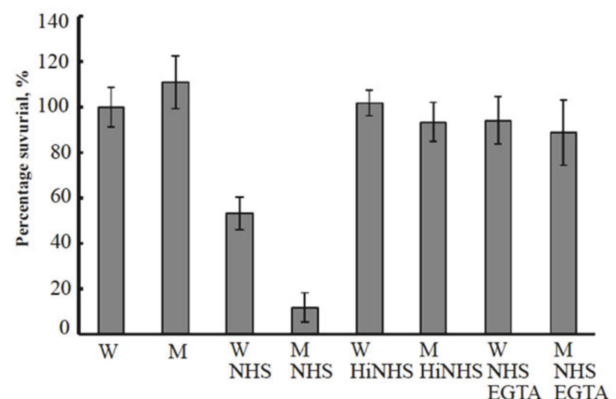

Fig. 7. Survival of *S. maltophilia* in adult NHS. Bacterial strains were mixed with an equal volume of healthy adult pooled serum, and viable cell numbers were determined by counting colonies after appropriately diluting and plating on LB agar plates. In some experiments, either heat-inactivated (Hi) or EGTA-pretreated (NHS+EGTA) serum was used. W and M represent *S. maltophilia* ATCC13637 and SmMopB, respectively.

The vaccine potential of MopB of *S. maltophilia*

In Gram-negative bacteria, OmpA and related proteins have been proposed for the design of vaccines against bacterial infection (Lee *et al.*, 2008; Maiti *et al.*, 2011; Pore and Chakrabarti, 2013). In the absence of adjuvant, most soluble foreign proteins induce a weak immune response (Mondino *et al.*, 1996); however, OmpA proteins from different species are capable of inducing specific immune and cytotoxic responses (Feng *et al.*, 2004; Lee *et al.*, 2008; Pore *et al.*, 2012; Hsieh *et al.*, 2013; Bhowmick *et al.*, 2014).

The criteria that make a protein suitable for vaccine development include exposure on the cell surface, sequence conservation, the presence of no more than one transmembrane membrane helix, and the likelihood of functioning as an adhesin (He *et al.*, 2010). We demonstrated that the *S. maltophilia* OmpA family member MopB 1) is exposed on the surface, constituting the most abundant OMP with a possible role in the maintenance of cell shape against harsh environment stresses, including human serum; 2) is highly conserved in strains of *Stenotrophomonas*; 3) lacks a transmembrane helix (analyzed by TMHMM server version 2.0 for the prediction of transmembrane helices in proteins; http://www.cbs.dtu.dk/services/TMHMM-2.0/); and 4) functions as an adhesin, as evidenced by the reduced adhesion ability of the SmMopB strain. Mutation of the *mopB* gene also significantly reduced cytotoxicity and increased serum sensitivity. Therefore, blocking the function of the most abundant protein MopB may be a novel way to control *S. maltophilia* infection. Taken together, our results suggest that MopB of *S. maltophilia* is a possible candidate for the development of vaccines to control the serious nosocomial infection of multidrug-resistant *S. maltophilia*, especially in immunosuppressed patients.

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