MacABCsm, an ABC-type tripartite efflux pump of Stenotrophomonas maltophilia involved in drug resistance, oxidative and envelope stress tolerances and biofilm formation

Yi-Tsung Lin1,2, Yi-Wei Huang3, Rung-Shiuan Liou3, Yi-Chih Chang4 and Tsuey-Ching Yang3*

1Division of Infectious Diseases, Department of Medicine, Taipei Veterans General Hospital, Taipei 112, Taiwan; 2School of Medicine, National Yang-Ming University, Taipei 112, Taiwan; 3Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei 112, Taiwan; 4Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 404, Taiwan

*Corresponding author. 155 Section 2, Lie-Nong Street, Taipei 112, Taiwan, ROC. Tel: +886-2-28267289; Fax: +886-2-28264092; E-mail: tcyang@ym.edu.tw

Received 16 April 2014; returned 11 June 2014; revised 25 June 2014; accepted 19 July 2014

Objectives: To characterize a five gene cluster, macRS-macABCsm, in Stenotrophomonas maltophilia.

Methods: The presence of macABCsm operon was verified by RT–PCR. The substrate spectrum of the MacABCsm efflux pump was investigated by mutant construction and susceptibility testing. The physiological role of MacABCsm was assessed by comparing the growth of wild-type and macABCsm mutant under different stresses. To examine the regulatory role of the two-component regulatory system (TCS) macRS in the expression of macABCsm operon, mutant construction, quantitative RT–PCR and susceptibility testing were employed.

Results: macAsm, macBsm and macCsm genes formed a three-membered operon. The MacABCsm efflux pump extruded macrolides, aminoglycosides and polymyxins and contributed to oxidative and envelope stress tolerances and biofilm formation. Inactivation of macRS TCS hardly influenced the expression of macABCsm operon and the antimicrobial susceptibility.

Conclusions: The MacABCsm pump has physiological roles in protecting S. maltophilia from the attack of oxidative and envelope stresses and in biofilm formation, which may be the reason why it can be constitutively expressed in the absence of antibiotics and is highly conserved in S. maltophilia isolates isolated from different environmental niches. However, the constitutive expression of macABCsm contributes to the intrinsic resistance of S. maltophilia to macrolides, aminoglycosides and polymyxins.

Keywords: bacteria, antibiotics resistance, physiological role

Introduction

Multidrug transporters are a diverse group of proteins, which can extrude noxious compounds and protect bacterial cells from environmental stresses. Based on sequence similarity, multidrug transporters are classified into five families: resistance–nodulation–cell division (RND); major facilitator superfamily (MFS); small multidrug resistance; multidrug, toxic compound extrusion; and ATP-binding cassette (ABC).1

ABC-type transporters, such as P-glycoprotein and MRP, were first reported in mammalian cells.2,3 The first bacterial ABC-type transporter, LmrA from Lactococcus lactis, was described in 1996.4 Interestingly, the ABC-type transporters seem to fulfil a major role in the drug resistance of pathogenic Gram-positive bacteria such as L. lactis, Lactobacillus brevis, Bacillus subtilis, Enterococcus faecalis, Oenococcus oeni and Streptococcus pneumoniae.5 Only very few members of ABC-type transporter families have been reported to be involved in drug resistance in Gram-negative bacteria, including MsbA of Escherichia coli, MacAB of E. coli, VcaM of Vibrio cholerae, MacAB of Neisseria gonorrhoeae, SmdAB of Serratia marcescens and SmrA of Stenotrophomonas maltophilia.6–11

The ABC-type transporters reported in Gram-positive and most Gram-negative bacteria are generally inner membrane transporters that can extrude compounds across the inner membrane. However, tripartite ABC-type efflux pumps consisting of an ABC-type inner membrane transporter, a membrane fusion protein and an outer membrane protein (OMP) have been subsequently reported recently, including MacAB-ToIC of E. coli, MacAB of N. gonorrhoeae, MacAB of Salmonella enterica serovar Typhimurium
(Salmonella Typhimurium) and PseEF of Pseudomonas syringae pv. syringae. 7,9,12,13

In addition to their roles in antimicrobial extrusion, the physiological impact of efflux pumps has attracted a lot of attention, especially for the constitutively expressed efflux pumps. The efflux pumps have been reported to participate in many physiological processes, such as virulence, pH homeostasis, alkali tolerance, cell division, membrane stress tolerance and oxidative stress adaption. 13–17

S. maltophilia, a non-fermentative Gram-negative bacterium, is a cause of opportunistic and nosocomial infections. It shows high-level intrinsic resistance to an array of antimicrobial agents. 18 Several tripartite efflux systems from the S. maltophilia chromosome have been characterized, including six RND-type efflux pumps (SmeABC, SmeDEF, SmeFLJ, SmeOP, SmeVWX and SmeYZ), an MFS-type efflux pump (EmrCABsm) and a fusaric acid-extrusion efflux pump (FuaABC). 19–25 In this study, a tripartite ABC-type efflux pump of S. maltophilia is reported for the first time. Our results demonstrate that MacABCsm plays a role in at least three important processes: it provides intrinsic resistance to aminoglycosides, macrolides and polymyxins; it contributes to oxidative and envelope stress tolerances; and it is involved in normal biofilm formation.

Materials and methods

Bacterial strains, primers and growth conditions

Table S1 (available as Supplementary data at JAC Online) summarizes the bacterial strains, plasmids and primers used in this study. For general purposes, bacterial strains were grown aerobically at 37°C in Luria–Bertani (LB) medium unless otherwise stated.

Construction of deletion mutants

Gene disruption was performed using a well-established procedure. 26 For plasmid construction, four PCR amplicons of different length, labelled 1–4 in Figure S1, were obtained by PCR using the primer sets MacAN-F/MacAN-R, MacCN-F/MacCN-R, MacCC-F/MacCC-R and MacSC-F/MacSC-R, respectively. The recombinant plasmid pΔMacAB was obtained by sequentially cloning amplicons 1 and 2 into pEX18Tc. Similarly, plasmid pΔMacC was derived from amplicons 2 and 3 and pΔMacRS from amplicons 4 and 1 (Figure S1). Plasmids pΔMacAB, pΔMacC and pΔMacRS were transported into S. maltophilia KJ by conjugation and the deletion mutants were selected as described previously. 26 The authenticity of the deletion mutants was checked by colony PCR amplification and sequencing.

Antimicrobial susceptibility testing

The susceptibilities of S. maltophilia strains to a number of antibiotics were tested by the agar dilution method on Mueller–Hinton agar (MH) according to CLSI guidelines.27 The MIC was defined as the lowest concentration tested by the agar dilution method on Mueller–Hinton agar (MH) according to CLSI guidelines.27 The MIC was defined as the lowest concentration tested by the agar dilution method on Mueller–Hinton agar (MH) according to CLSI guidelines.27 The MIC was defined as the lowest concentration tested by the agar dilution method on Mueller–Hinton agar (MH) according to CLSI guidelines.27 The MIC was defined as the lowest concentration tested by the agar dilution method on Mueller–Hinton agar (MH) according to CLSI guidelines.27 The MIC was defined as the lowest concentration tested by the agar dilution method on Mueller–Hinton agar (MH) according to CLSI guidelines.27

RT–PCR

DNA-free bacterial RNA was prepared as described previously.26 The primer MacC-F (Figure S1 and Table S1), which annealed with the 3′-end of the macC transcript, was used to generate cDNA. The resultant cDNA was subjected to PCR using the primers MacAQ-F/R and MacBQ-F/R (Table S1). An RT-PCR control of the RNA without reverse transcription was included to assure that there was no DNA contamination. Amplicons were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Stress challenge assays

Overnight cultures of KJ and KJΔMacAB were subcultured into fresh LB, containing a different stressor, with an OD450 of 0.15. H2O2 (2 mM), menadione (16 μg/mL) and Triton X-100 (200 mg/mL) were used for the evaluation of oxidative and envelope stresses. A non-treated control was also prepared without any additives. Cells were grown aerobically and the OD450 was measured every 3 h.

SDS survival analysis

The LB-cultured cells grown to stationary phase were adjusted to an OD450 of 1.0 with fresh LB. The cells were treated with or without 0.02% SDS for 10 min and the final OD450 was measured. The percentage survival was defined as the OD450 ratio of the SDS group to the SDS-free counterpart. Each experiment was repeated at least three times.

Disc diffusion assay

A bacterial cell suspension of 10⁷ cells/mL was streaked onto MH plates. A 300 U of polymyxin B disc and 10 mg of polymyxin E disc were placed onto the agar surface. The diameter of the zone of inhibition was measured (mm) after 24 h incubation at 37°C. Each experiment was repeated at least three times.

Assays of biofilm formation

Overnight cultures grown in LB medium were adjusted to an OD450 of 0.1 and used as the inoculum. Each microtiter well of 96-well polystyrene chloroform-microlitre plates was then inoculated with 200 μL of the OD450 0.1 inoculum, with a minimum of three wells per bacterial strain for each assay, and incubated at 37°C for 48 h. The total cell biomass was estimated by measuring the OD450 using a plate reader. Then, cultures were decanted and wells were washed with distilled water and 200 μL of 1% crystal violet was added to the wells for 15 min. The stained biofilms were rinsed with distilled water and extracted with 200 μL of 70% ethanol. The amount of biofilm was quantified by measuring the A570 of dissolved crystal violet. Uninoculated medium controls were included. The levels of biofilm were expressed relative to the final cell density measured prior to biofilm assay (A570/OD450).

RNA preparation and quantitative real-time PCR (qRT–PCR)

DNA-free RNA was converted into cDNA using the MMLV Reverse Transcriptase 1st Strand cDNA Synthesis Kit (Epicentre Biotechnologies, Taiwan) with random hexamers as primers. The cDNA was then used directly as a template for qRT–PCR. qRT–PCR was accomplished using Smart Quant Green Master Mix (Protech Technology Enterprise Co.). The primers used for qRT–PCR are listed in Table S1. The 16S rRNA gene was chosen as the normalizing gene. Relative quantities of mRNA from each gene of interest were determined by the comparative cycle threshold method.28 Each experiment was performed in triplicate.

Results and discussion

Analysis of the macRS-macABCsm cluster in S. maltophilia

The macrolide-specific tripartite ABC-type efflux transporter MacAB-TolC has been identified in E. coli. 7 A homologue of MacA-MacB ABC-type efflux systems was identified in S. maltophilia K279a through NCBI BlastP search (Figure S1). Smlt1539 (named as...
macAsm) and Smlt1538 (named as macBsm) genes in *S. maltophilia* K279a overlapped by 4 bp. Smlt1537 (annotated as macCsm hereafter), which encoded a putative OMP, was located down- stream macBsm. The macBsm and macCsm genes were separated by 5 bp only (Figure S1). macAsm encoded a predicted 414 aa membrane fusion protein, which showed 39% identity to MacA of *E. coli* and 42% identity to MacA of *N. gonorrhoeae*. The 652 aa MacBsm contained a nucleotide-binding cassette domain and ABC signature sequence in its N-terminal half as well as an inner membrane domain of four predicted transmembrane regions in its C-terminal half, a typical organization of ABC-type transporters.29 MacBsm was 57% identical to MacBs of *E. coli* and *N. gonorrhoeae*.

A putative two-component regulatory system (TCS), Smlt1540 and Smlt1541 (named as macR and macS hereafter), was divergently located upstream of the macABCsm cluster (Figure S1). The macS gene encodes a 469 aa sensor kinase. MacS is assumed to be co-transcribed with macR, which encodes the response regulator MacR. MacS has a predicted short cytoplasmic part (amino acid residues 1–11) followed by a transmembrane helix (amino acid residues 12–34) and an extracellular region (amino acid residues 35–469), which probably serves as the sensor. MacR has two domains: an N-terminal signal receiver domain (amino acid residues 10–120) and a C-terminal effector domain (amino acid residues 140–230).

**MacABCsm forms an operon**

RT–PCR was used to prove the presence of the macABCsm operon. The RNA from the log-phase bacterial culture of wild-type *S. maltophilia* KJ was prepared. The primer MacC-C, which was annealed onto the macCsm transcript (Figure S1 and Table S1), was used to generate cDNA. The cDNA was subjected to PCR using primers MacAQ-F/MacAQ-R and MacBQ-F/MacBQ-R, both of which were annealed to macAsm and macBsm, respectively (Figure S1 and Table S1). The products with expected sizes of 267 and 220 bp were obtained, indicating that the macAsm, macBsm and macCsm genes were co-transcribed and the macABCsm operon was intrinsically expressed.

**Role of MacABCsm efflux pump in antibiotic resistance**

To examine the contribution of the MacABCsm pump to antibiotic resistance, the macAsm deletion mutant, KJ ΔMacAB, was constructed. Compared with wild-type KJ, disruption of macB slightly decreased the susceptibility to gentamicin, kanamycin, erythromycin, rokitamycin and spiramycin as well as significantly lowered the MICs of amikacin, tobramycin, leucomycin, polymyxin B and polymyxin E (Table 1). The susceptibilities to chloramphenicol, quinolone and tetracycline were observed to be similar between strains KJ and KJ ΔMacAB (Table 1). Therefore, the MacABCsm pump contributes to intrinsic aminoglycoside, macrolide and polymyxin resistance in *S. maltophilia*.

To test whether the MacCsm protein is the sole competent OMP for the function of the MacABCsm pump, a ΔmacCsm allele was introduced into the wild-type strain, yielding mutant KJ ΔMacC. Interestingly, deletion of macCsm reduced the MICs, but failed to compromise resistance to the same extent that deletion of macBsm did (Table 1, KJ ΔMacC versus KJ ΔMacAB), indicating that in addition to MacC, MacAB may associate with other OMPS to form a functional tripartite pump. TolCsm, a cognate OMP for various tripartite pumps, has been characterized in our recent publication. Specifically, inactivation of tolCsm has compromised resistance to a variety of agents, including aminoglycosides and macrolides.30 Therefore, TolCsm is likely the compatible OMP for MacAB.

**Physiological roles of MacABCsm efflux pump**

Inactivation of macAB compromised the resistance to aminoglycosides, macrolides and polymyxins indicating that the MacABCsm pump is intrinsically expressed in wild-type KJ. An intrinsically functional efflux pump may have other physiological roles in addition to drug extrusion.31 To consider the roles of the MacABCsm efflux pump in physiology and the environmental stress response, bacterial growth and the resistance levels of wild-type KJ and isogenic KJ ΔMacAB against different stresses were determined. The colony morphology and bacterial growth kinetics of the macAB mutant were indistinguishable from those of the wild-type in LB medium (Figure 1a). The growth characteristics of KJ and KJ ΔMacAB were then determined over a period of 24 h in LB medium with different stresses and temperatures. The conditions tested included oxidative stress (16 mg/L menadione and 2 mM H2O2), envelope stress (200 mg/L Triton X-100) and temperatures of 25°C and 40°C. Figure 1 demonstrates that the logarithmic growth of KJ ΔMacAB was compromised in the presence of oxidative stress, particularly in the presence of menadione (Figure 1b). Furthermore, the growth of KJ ΔMacAB was significantly impaired in the presence of Triton X-100 (Figure 1c). However, the growth of the macABC mutant was indistinguishable from the growth of the wild-type at the different temperatures.

<table>
<thead>
<tr>
<th>Compound</th>
<th>KJ</th>
<th>KJ ΔMacAB</th>
<th>KJ ΔMacC</th>
<th>KJ ΔMacRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloramphenicol</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Quinolones</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>nalidixic acid</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>norfloxacin</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>doxycycline</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>tetracycline</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>1024</td>
<td>256</td>
<td>512</td>
<td>1024</td>
</tr>
<tr>
<td>amikacin</td>
<td>1024</td>
<td>512</td>
<td>1024</td>
<td>1024</td>
</tr>
<tr>
<td>gentamicin</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>kanamycin</td>
<td>512</td>
<td>64</td>
<td>64</td>
<td>512</td>
</tr>
<tr>
<td>tobramycin</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>Macrolides</td>
<td>64</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>erythromycin</td>
<td>256</td>
<td>16</td>
<td>128</td>
<td>256</td>
</tr>
<tr>
<td>leucomycin</td>
<td>512</td>
<td>256</td>
<td>512</td>
<td>512</td>
</tr>
<tr>
<td>rokitamycin</td>
<td>2048</td>
<td>1024</td>
<td>1024</td>
<td>2048</td>
</tr>
<tr>
<td>spiramycin</td>
<td>2048</td>
<td>1024</td>
<td>1024</td>
<td>2048</td>
</tr>
<tr>
<td>Polymyxins</td>
<td>4</td>
<td>0.5</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>polymyxin B</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>polymyxin E</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>
Figure 1. Growth curves of *S. maltophilia* strains KJ and KJΔMacAB. Growth of *S. maltophilia* strains KJ and KJΔMacAB in (a) LB broth, (b) LB broth containing 16 mg/L menadione or 2 mM H₂O₂ and (c) LB broth containing 200 mg/L Triton X-100. The growth was monitored by recording the OD₄₅₀. Each data point is the mean of three independent cultures and error bars indicate the standard deviation. Significance at each timepoint assayed was statistically analysed by Student’s t-test. *P ≤ 0.05.*
The impact of MacABCsm on the envelope stress tolerance was further tested by polymyxin susceptibility and SDS survival assay. The macAB mutant was more susceptible to polymyxin and SDS than wild-type KJ (Table 1, Figure 2). These results support that the MacABCsm pump has a physiological role in oxidative and envelope stress tolerances.

The crystal violet biofilm assay was employed to determine the ability of wild-type KJ and mutants KJΔmacAB and KJΔmacC to form biofilms. KJΔmacAB (3.33 ± 1.0) and KJΔmacC (2.05 ± 0.7) had 48% and 68% reduction in the biofilm formation compared with wild-type KJ (6.42 ± 1.6).

**Inactivation of MacRS hardly affected the expression of macABCsm operon**

The macRS operon is divergently transcribed from the macABC operon, strongly suggesting its regulatory role in macABCsm. Therefore, we constructed a macRS mutant, KJΔmacRS, to assess the underlying regulatory mechanism of MacRS TCS. Surprisingly, strains KJ and KJΔmacRS displayed similar susceptibilities to all the antimicrobials tested (Table 1), suggesting that the macRS TCS is less relevant to the expression of the macABCsm operon. To further verify this, transcripts of macAsm, macBsm and macCsm in strains KJ and KJΔmacRS were compared by qRT–PCR. Indeed, macA, macB and macC transcripts in both strains were comparable (Figure S2), supporting that expression of the macABCsm operon is not altered in the case of macRS inactivation.

Since only limited numbers of tripartite ABC-type efflux pumps have been studied so far, their regulation is less documented. The only example is that the macAB of *Salmonella Typhimurium* is negatively regulated by a TCS PhoP-PhoQ. The PhoP-PhoQ is not located in the flanking region of the macAB operon in *Salmonella Typhimurium*. Based on the general perception, the genetic organization of macRS and macABCsm operons strongly suggests that MacRS should play a critical role in the expression of the macABCsm operon. However, our results are discordant with the anticipation. Two possibilities were proposed to explain these observations: (i) the macABCsm operon is indeed not subjected to the regulation of macRS TCS under the conditions we tested; and (ii) MacRS is not the sole regulatory system involved in the expression of the macABCsm operon. The existence of additional regulatory pathway(s) for macABCsm operon expression compensates the function of MacRS when macRS TCS is inactivated.

**Conclusions**

MacAB homologues have been reported in *E. coli*, *N. gonorrhoeae* and *Salmonella Typhimurium*. **MacAB of *E. coli*** mainly participates the extrusion of macrolides; however, the role of MacAB in macrolide efflux is less significant in *N. gonorrhoeae* and *Salmonella Typhimurium*. MacAB of *Salmonella Typhimurium* has been proven to be involved in virulence and oxidative stress adaption. **The MacABCsm of *S. maltophilia*** reported in this article is different from the MacAB homologues of other microorganisms: (i) the MacABCsm pump has its own cognate OMP, MacCsm; (ii) the macABCsm operon is intrinsically expressed; (iii) compared with MacAB-TolC of *E. coli*, MacABCsm exhibits a wider substrate spectrum for the extrusion of macrolides, aminoglycosides and polymyxins; and (iv) compared with MacAB of *Salmonella Typhimurium*, MacABCsm has a variety of physiological functions in envelope and oxidative stress tolerances as well as biofilm formation.

*S. maltophilia* is ubiquitous in nature, including in soil, water, plants and animals. The physiological function of MacABCsm has positive impacts on fitness in settings that are part of the life cycle of *S. maltophilia*. For example, MacABCsm may support the ability of *S. maltophilia* to tolerate envelope stresses caused by membrane-disrupting disinfectants or detergents widely distributed in nature and underpin the oxidative stress tolerance of *S. maltophilia*. The contribution of MacABCsm to biofilm formation may help *S. maltophilia* to face strict environmental challenges. There are many *S. maltophilia* genome sequences published in the NCBI database and the macABCsm operon is well conserved in all available *S. maltophilia* genomes. The physiological roles of MacABCsm in the fitness benefits may be the reason why macABCsm can be constitutively expressed and stably conserved in different *S. maltophilia* isolates isolated from different

![Figure 2](https://academic.oup.com/jac/article-abstract/69/12/3221/824005)
environmental niches. This outcome makes significant impacts on the intrinsic resistance of *S. maltophilia* to macrolides, aminoglycosides and polymyxins.

**Funding**

This research was supported by Ministry of Science and Technology (NSC 101-2320-B-010-053-MY3) and China Medical University (CMU100-TC-04).

**Transparency declarations**

None to declare.

**Supplementary data**

Table S1 and Figures S1 and S2 are available as Supplementary data at [Supplementary data](http://jac.oxfordjournals.org/).

**References**