The SmeYZ Efflux Pump of *Stenotrophomonas maltophilia* Contributes to Drug Resistance, Virulence-Related Characteristics, and Virulence in Mice

Yi-Tsung Lin,^{a,b} Yi-Wei Huang,^c Shiang-Jiuun Chen,^d Chia-Wei Chang,^c Tsuey-Ching Yang^c

Division of Infectious Diseases, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan^a; School of Medicine, National Yang-Ming University, Taipei, Taiwan^b; Department of Biotechnology and Laboratory Science in Medicine, National Yang-Ming University, Taipei, Taiwan^c; Department of Life Science, Institute of Ecology and Evolutionary Biology and TechComm-5, College of Life Science, National Taiwan University, Taipei, Taiwan^d

The resistance-nodulation-division (RND)-type efflux pump is one of the causes of the multidrug resistance of *Stenotrophomonas maltophilia*. The roles of the RND-type efflux pump in physiological functions and virulence, in addition to antibiotic extrusion, have attracted much attention. In this study, the contributions of the constitutively expressed SmeYZ efflux pump to drug resistance, virulence-related characteristics, and virulence were evaluated. *S. maltophilia* KJ is a clinical isolate of multidrug resistance. The *smeYZ* isogenic deletion mutant, KJΔYZ, was constructed by a gene replacement strategy. The antimicrobial susceptibility, virulence-related physiological characteristics, susceptibility to human serum and neutrophils, and *in vivo* virulence between KJ and KJΔYZ were comparatively assessed. The SmeYZ efflux pump contributed resistance to aminoglycosides and trimethoprim-sulfamethoxazole. Inactivation of *smeYZ* resulted in attenuation of oxidative stress susceptibility, swimming, flagella formation, biofilm formation, and secreted protease activity. Furthermore, loss of SmeYZ increased susceptibility to human serum and neutrophils and decreased *in vivo* virulence in a murine model. These findings suggest the possibility of attenuation of the resistance and virulence of *S. maltophilia* with inhibitors of the SmeYZ efflux pump.

Stenotrophomonas maltophilia, a widespread environmental microorganism, is an emerging multidrug-resistant global opportunistic pathogen. *S. maltophilia* is generally associated with septicemia and various infections, especially respiratory infections, in immunocompromised patients and cystic fibrosis (CF) patients. The difficulty in the treatment of *S. maltophilia* infection has been attributed to its intrinsic multidrug resistance (1). The clinical isolates of *S. maltophilia* generally exhibit high resistance to β-lactams, macrolides, and aminoglycosides, which is attributed to the overexpression of β-lactamases, aminoglycoside-modifying enzymes, and multidrug efflux pumps (2).

The multidrug efflux pumps are membrane proteins that are involved in the extrusion of antibiotics and are classified into five families, including resistance-nodulation-division (RND), major facilitator superfamily (MFS), small multidrug resistance (SMR), ATP-binding cassette (ABC), and multidrug and toxic compound extrusion (MATE) (3). Of the five families, the RND-type efflux pumps form a tripartite assembly in the bacterial membrane, comprising an RND-type inner membrane protein (IMP), an outer membrane protein (OMP), and a membrane fusion protein (MFP) to link the IMP and OMP (4). RND-type efflux pumps confer resistance to a large array of drugs and make a significant contribution to drug resistance in Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *S. maltophilia*.

In addition to the known roles in antibiotic extrusion, RND-type efflux pumps are increasingly recognized as components linking bacterial physiology and virulence (5). For example, inhibition of the efflux pumps impairs biofilm formation in *Salmonella enterica* serovar Typhimurium (6). The BpeAB-OprB pump of *Burkholderia pseudomallei*, the AcrAB-TolC pump of *Enterobacter cloacae*, and the AcrAB-TolC pump of *Yersinia pestis* are involved in virulence (7–9). In addition, evidence that the expression of efflux pumps is regulated by different physiological stresses

further indicates the close relationship between RND-type efflux pumps and physiological functions. The MexXY efflux pump of *P. aeruginosa* (10, 11) and the CmeABC efflux pump of *Campylobacter jejuni* (12) are induced by oxidative stress. The MexEF-OprN efflux pump of *P. aeruginosa* (13) is activated in response to oxidative and nitrosative stresses. A variety of membrane-damaging agents can induce the MexCD-OprJ efflux pump of *P. aeruginosa* (14).

Eight putative RND-type efflux systems (SmeABC, SmeDEF, SmeGH, SmeIJK, SmeMN, SmeOP, SmeVWX, and SmeYZ) were annotated in the *S. maltophilia* K279a genome (15). Of the eight pumps, six pumps (SmeABC, SmeDEF, SmeIJK, SmeOP, SmeVWX, and SmeYZ) have been characterized, mainly focusing on their roles in antimicrobial extrusion (16–21). In particular, it was noted that the SmeIJK and SmeYZ pumps are constitutively highly expressed, and both are redundant in extrusion of aminoglycosides (18, 21). It is thought that antimicrobial extrusion may not be the major function of the SmeIJK and SmeYZ pumps, and both pumps may have distinct physiological functions. Recently, we reported that the physiological role of the

Received 13 February 2015 Returned for modification 6 March 2015
Accepted 20 April 2015

Accepted manuscript posted online 27 April 2015

Citation Lin Y-T, Huang Y-W, Chen S-J, Chang C-W, Yang T-C. 2015. The SmeYZ efflux pump of *Stenotrophomonas maltophilia* contributes to drug resistance, virulence-related characteristics, and virulence in mice. *Antimicrob Agents Chemother* 59:4067–4073. doi:10.1128/AAC.00372-15.

Address correspondence to Tsuey-Ching Yang, tcyang@ym.edu.tw.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.
doi:10.1128/AAC.00372-15

SmeIJK pump is linked to the maintenance of membrane integrity (18). In this article, we further elucidated the significance of the SmeYZ pump in physiological functioning. The results demonstrated that inactivation of the SmeYZ pump compromised the virulence-related physiological functions of swimming, flagella formation, oxidative stress susceptibility, biofilm formation, and protease secretion and thus decreased *in vivo* virulence. These findings supported the possibility that SmeYZ can develop as a new target for the treatment of *S. maltophilia* infection.

MATERIALS AND METHODS

Bacterial strains, primers, and growth conditions. *S. maltophilia* KJ is a clinical isolate (22). All primers used in this study were designed based on the *S. maltophilia* K279a genome sequence (15). For general purposes, strains were grown aerobically in Luria-Bertani (LB) medium except as specifically noted.

Construction of deletion mutant KJΔYZ. To construct the Δ*smeYZ* mutant, the 1,759-bp product containing partial *smeYZ* genes was amplified from the genome of *S. maltophilia* KJ using primers SmeYZ-F (5'-C GCAAGCTTGACCTGCGCTATGCC-3') and SmeYZ-R (5'-TGCGAAT TCCAGCAGCATCGCCTCC-3') and cloned into pEX18Tc, generating pYZ. An internal 489-bp ClaI-ClaI DNA fragment was removed from pYZ, obtaining pΔYZ. The plasmid pΔYZ was introduced into *E. coli* S17-1 by transformation and mobilized into *S. maltophilia* KJ via conjugation (23). Transconjugants carrying deleted *smeYZ* in the chromosome were obtained by two-step selection on LB agar containing tetracycline (30 μg/ml)-norfloxacin (2.5 μg/ml) and then on LB agar containing 10% (wt/vol) sucrose. Mutants were confirmed by colony PCR (24).

Antimicrobial susceptibility test. The antimicrobial susceptibilities of *S. maltophilia* strains were tested by the serial 2-fold dilution method in Mueller-Hinton (MH) agar according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (25). The plates were incubated at 37°C and examined visually after 24 h. The MIC was defined as the lowest concentration of antimicrobial agent that inhibited visible growth. All antibiotics were purchased from Sigma-Aldrich (USA).

Growth kinetic assay. Overnight cultures were reinoculated into fresh LB medium with an initial optical density at 450 nm (OD₄₅₀) of 0.15. The OD₄₅₀ readings were taken every 3 h for a total time of 24 h.

Cell surface hydrophobicity assay. The cell surface hydrophobicity values were determined using a modified two-phase partitioning system (26). The logarithmic-phase cells were washed twice with phosphate-buffered saline (PBS) buffer and resuspended in PBS before the OD₄₅₀ values were recorded (A₀). Bacterial partitioning was done by addition of 5 ml of bacterial suspension to 1 ml of hexadecane. The bacterial suspension in the phase system was vortexed for 2 min and allowed to stand undisturbed for phase separation for 3 min at room temperature. The lower aqueous phase was read to determine the OD₄₅₀ (A). The hydrophobicity value was calculated as $[1 - (A/A_0)] \times 100$.

H₂O₂ susceptibility test (disk diffusion assay). The H₂O₂ susceptibility was tested as described previously with some modifications (27). MH plates were streaked with a cotton swab soaked in *S. maltophilia* cell suspension of 10⁷ cells/ml. The sterile paper disks (6 mm in diameter) were placed on the plate, and the disks were spotted with 20 μl of 10% H₂O₂. The diameter of the zone of growth inhibition around each disk was measured after a 24-h incubation at 37°C.

Menadione susceptibility assay. Overnight cultures of the tested strains were diluted to an OD₄₅₀ of 0.15 with LB broth. Cells were grown for 2 h, and serial dilutions were plated onto LB medium containing 0, 10, 20, and 30 μg/ml menadione. Plates were incubated for 24 h at 37°C and scored for CFU. The percentage of survival was defined as the CFU ratio of KJΔYZ to wild-type KJ.

Swimming assay. To assess swimming motility, 5 μl of overnight culture cultivated at 37°C in LB broth was inoculated at the swimming agar surface (1% tryptone, 0.5% NaCl, and 0.15% agar) (28). The plates were

incubated at 37°C for 48 h. Results are expressed as diameters (millimeters) of swimming zones.

Flagella staining. The presence of flagella was determined by negative staining and transmission electron microscopy (TEM) (Hitachi H-7650 microscope). Bacteria were negatively stained using the drop method for 10 s with 1% phosphotungstic acid (pH 7.4) on Formvar-coated copper grids (29).

Assays of biofilm formation. The biofilm assay was carried out as described previously with some modifications (30). The overnight-cultured bacteria were adjusted to an OD₄₅₀ of 0.1, and 200 μl of bacterial suspension was inoculated into each microtiter well, with a minimum of three wells per bacterial strain for each assay. After a 48-h incubation at 37°C, the total cell biomass was estimated by enumerating the CFU. The culture supernatants were decanted, and the wells were washed with distilled water. The biofilms in the well were stained by 200 μl of 1% crystal violet for 15 min. The stained biofilms were rinsed with distilled water, extracted with 200 μl of 70% ethanol, and quantified by measuring the A₅₇₀ of dissolved crystal violet. Uninoculated medium controls were included. The levels of biofilm formation were expressed as the crystal violet staining relative to the cell biomass (A₅₇₀/CFU).

Secreted protease activity assay. The secreted protease activity of the bacteria was assayed using LB agar containing 1% skim milk. For the convenience of bacterial loading, the skin milk agar was prepared with a 6-mm-diameter hole in the center. Strains tested were cultured in LB agar at 37°C for 20 h and adjusted with LB broth to an OD₄₅₀ of 1.0, and 40 μl of these cultures was dripped onto the hole of the skim milk agar plates. After incubation at 37°C for 72 h, the proteolytic activity of bacteria was assessed by measuring the transparent zones around the bacteria.

Serum killing assays. Serum killing assays were performed according to the published method with some modifications (31). The survival of exponential-phase bacteria (wild-type KJ and mutant KJΔYZ) in nonimmune human serum was measured. A log-phase inoculum of 5×10^4 CFU was mixed at a 1:1 (vol/vol) ratio with mixed nonimmune human serum or heat-inactivated serum (56°C for 30 min) donated by 2 healthy volunteers. The final mixture, comprising 50% nonimmune serum or heat-inactivated serum by volume, was incubated at 37°C for 1 to 3 h. To determine the number of viable bacteria after exposure to serum, an aliquot of each bacterial suspension was removed at the designated time point, diluted 10-fold by addition of Mueller-Hinton broth, and plated on Mueller-Hinton agar. The mean survival ratio was plotted.

Killing assays by human neutrophils. Human neutrophils were freshly isolated from peripheral blood donated by 2 healthy volunteers. An inoculum containing 10⁵ CFU of bacteria was opsonized with 25% normal human serum for 15 min on ice and incubated with or without 10⁵ human neutrophils in 1× phosphate-buffered saline at 37°C for 45 min. The percentages of survival of wild-type KJ and mutant KJΔYZ were calculated on the basis of the viable counts relative to those for the neutrophil controls (32).

Mouse inoculation experiments. Virulence was evaluated by mortality in a murine model of septicemia generated by intraperitoneal injection (33). Groups of 8-week-old male C57BL/6 mice were infected intraperitoneally with the wild-type KJ and mutant KJΔYZ (10⁹ CFU; 5 mice for each dose). The exact inoculation dose was confirmed by serial dilution and plating on Mueller-Hinton agar. Mice were monitored for 14 days. A control group without bacterial injection was also included. All animal care procedures and protocols were approved by the Institutional Animal Care and Use Committee of National Yang-Ming University.

Statistical analyses. Data are presented as means ± standard deviations. The statistical significance was assessed by a 2-tailed Student's *t* test using Prism 5 (GraphPad) software. Survival was assessed by Kaplan-Meier analysis with a log rank test. *P* values of <0.05 were considered significant.

RESULTS

Loss of SmeYZ affects susceptibility to aminoglycosides and SXT. Because the SmeYZ is an RND-type efflux pump, the con-

TABLE 1 Susceptibility tests of *S. maltophilia* KJ and KJΔYZ

Antimicrobial	MIC (μg/ml)	
	KJ	KJΔYZ
β-Lactams		
Ampicillin	>2,048	>2,048
Piperacillin	1,024	1,024
Cefuroxime	2,048	>2,048
Cefoxitin	1,024	1,024
Aztreonam	>2,048	>2,048
Chloramphenicol	8	8
Nalidixic acid	8	8
Tetracycline	16	8
Aminoglycosides		
Amikacin	1,024	8
Gentamicin	1,024	8
Kanamycin	256	8
Macrolides		
Erythromycin	64	128
Leucomycin	256	128
Trimethoprim-sulfamethoxazole	32/160	2/10

tribution of SmeYZ to antimicrobial susceptibility was first evaluated. The *smeYZ* mutant (KJΔYZ) was more susceptible to the aminoglycosides (amikacin, gentamicin, and kanamycin) (Table 1), which is consistent with the previous findings (21). In addition, deletion of SmeYZ compromised resistance to trimethoprim-sulfamethoxazole (SXT) (Table 1).

Loss of SmeYZ affects virulence-related physiological characteristics of *S. maltophilia* KJ. The bacterial growth was assessed by monitoring the OD₄₅₀ and viable counts at the different growth time points. The logarithmic-phase growth of KJΔYZ was comparable to that of wild-type KJ, but the OD₄₅₀ of KJΔYZ cells gradually declined after 9 h of culture (Fig. 1A). We also noticed that the KJΔYZ cells were gradually aggregated onto the surface of the culture bottle after 9 h. However, Fig. 1B demonstrates that the viable cells of KJ and KJΔYZ were comparable during the growth course monitored. In addition, we also noticed that the bacterial colony of KJΔYZ was homogeneously smaller than that of KJ when a single colony was isolated by the three-phase streaking method on LB agar (Fig. 1C). To further address whether the bacterial surface characteristics affect cell stacking, the cell surface hydrophobicity values of KJ and KJΔYZ were determined. Figure 1D shows that the hydrophobicity value of KJΔYZ was higher than that of KJ.

The role of SmeYZ in the response to oxidative stresses was tested by H₂O₂ and menadione susceptibility assays. The mean zone of

FIG 1 Bacterial growth curves, viability, and colony sizes. (A) Growth curves. The overnight-cultured bacteria were inoculated into fresh LB broth at the initial OD₄₅₀ of 0.15. The bacterial growth was monitored by recording the OD₄₅₀ every 3 h. Data are the means from three independent experiments. Error bars indicate the standard deviations for three triplicate samples. (B) Bacterial viability was monitored by counting the numbers of CFU. Data are the means from three independent experiments. Error bars indicate the standard deviations for three triplicate samples. (C) Single colony isolation. The single colonies of KJ and KJΔYZ cells were isolated by the three-phase streaking method on LB agar. (D) Bacterial surface hydrophobicity values. The bacterial surface hydrophobicity values were determined by partitioning of cells in two-phase systems (water-hexadecane). Data are the means from three independent experiments. Error bars indicate the standard deviations for three triplicate samples. *, $P < 0.05$, significance calculated by Student's t test.

FIG 2 Oxidative stress susceptibility assay. Data are the means from three independent experiments. Error bars indicate the standard deviations for three triplicate samples. *, $P < 0.05$, significance calculated by Student's t test. (A) H₂O₂ susceptibility. The MH agar was uniformly spread with bacterial cell suspension. Sterile filter paper with 20 μ l of 10% H₂O₂ was placed onto MH agar, and the diameter of a zone of growth inhibition was measured after 24 h of incubation at 37°C. (B) Menadione tolerance assay. Serial dilutions of the logarithmic-phase bacterial cells were plated onto LB containing 0, 10, 20, and 30 μ g/ml menadione. The CFU were scored after 24 h of incubation at 37°C. The percentage of relative survival was defined as the CFU ratio of mutant to wild type.

H₂O₂ inhibition exhibited by KJΔYZ (55 ± 3.0 mm) was larger than that produced by the parental strain KJ (45 ± 3.2 mm) (Fig. 2A). Furthermore, the survival rates of strains KJ and KJΔYZ were determined by the plate count method after the strains tested were exposed to different concentrations of menadione. The viability of KJΔYZ relative to that of KJ decreased when the menadione concentration was increased (Fig. 2B), supporting the observation that KJΔYZ is more susceptible to oxidative stress than wild-type KJ.

The swimming abilities of KJ and KJΔYZ cells for migration through semisolid agar (0.15% agar) were evaluated. After 48 h of culture, KJ cells exhibited a swimming zone of 32 ± 2 mm. In contrast, no swimming zone was observed for KJΔYZ cells except the KJΔYZ grew a lawn of approximately 6 mm (Fig. 3A). Since the flagella are the critical organelles determining bacterial motility, the impact of *smeYZ* on flagella formation was examined. Electron microscopy analyses showed that wild-type KJ cells had several flagellar structures in the pole region. In contrast, no flagella were observed in KJΔYZ cells (Fig. 3B).

FIG 3 Motility ability and flagella formation. (A) Motility ability. Five microliters of bacterial cell suspension was inoculated into the swimming agar (1% tryptone, 0.5% NaCl, and 0.15% agar). Results were expressed as diameters (millimeters) of swimming zones after 48 h of incubation at 37°C. Data are the means from three independent experiments. Error bars indicate the standard deviations for three triplicate samples. *, $P < 0.05$, significance calculated by Student's t test. (B) Flagella formation. The flagella were negatively stained with 1% phosphotungstic acid (pH 7.4) and observed by TEM.

The crystal violet biofilm assay was used to determine the ability of KJ and KJΔYZ strains to form biofilms. The statistical analysis revealed that KJΔYZ had a reduced ability to form biofilm in comparison to the wild-type KJ (Fig. 4A). The secreted protease activity was determined by measuring the transparent zones around the bacteria. The transparent zone of KJΔYZ was smaller than that of wild-type KJ (Fig. 4B), indicating that KJΔYZ exhibited a lower secreted protease activity.

Loss of *smeYZ* affects susceptibility to human serum and neutrophils. To compare the sensitivity to the serum's bactericidal effect between wild-type KJ and mutant KJΔYZ cells, serum killing assays were performed. Heat-inactivated serum was used as the control, and no killing effects were shown (data not shown). Killing of the KJΔYZ cells by nonimmunized healthy human serum was significantly more efficient than that of KJ cells at the designated time point (Fig. 5A).

We then analyzed neutrophil-mediated killing of wild-type KJ and KJΔYZ cells. Compared to those for KJ cells, the survival numbers for KJΔYZ cells were markedly reduced after incubation with human neutrophils for 45 min (Fig. 5B). Thus, the *SmeYZ* pump of *S. maltophilia* confers protection again, not only for serum killing but also for neutrophil-mediated killing.

Loss of *smeYZ* affects virulence to mice. Finally, the virulence *in vivo* of KJ and KJΔYZ cells was compared using the murine model of septicemia generated by intraperitoneal injection

FIG 4 Biofilm formation and secreted protease activity assay results. Data are the means from three independent experiments. Error bars indicate the standard deviations for three triplicate samples. *, $P < 0.05$, significance calculated by Student's t test. (A) Biofilm formation. Each microtiter well was inoculated with 200 μ l of the OD₄₅₀ 0.1 bacterial culture and incubated at 37°C for 48 h. The total cell biomass was estimated by enumerating the CFU. The amount of biofilm was determined by crystal violet staining. The stained biofilms were quantified by measuring the A_{570} of dissolved crystal violet. (B) Secreted protease activity assay. Forty microliters of bacterial cell suspension was dipped onto LB agar containing 1% skim milk. After incubation at 37°C for 72 h, the proteolytic activity of the bacteria was assessed by measuring the transparent zones around the bacteria.

(Fig. 6). Upon intraperitoneal infection of mice, KJΔYZ cells were less virulent than KJ cells. The survival rates of mice following infection with KJ and KJΔYZ strains were 22.2% and 77.8%, respectively ($P = 0.0659$).

DISCUSSION

Crossman et al. have reported that SmeYZ contributes to the extrusion of aminoglycosides (15). In this study, we further demonstrated that SXT is also a substrate for the SmeYZ pump in addition to aminoglycosides. Historically, SXT is considered the first line of defense in *S. maltophilia* infection. The known underlying mechanism responsible for SXT resistance in *S. maltophilia* is the gain in the *sul1* resistance gene of class 1 integrons and/or the *sul2* resistance gene of the insertion element (34, 35). The finding in this article is the first report linking the RND-type efflux pump to SXT resistance in *S. maltophilia*.

A smaller single colony in LB agar was observed for KJΔYZ cells than for wild-type KJ cells (Fig. 1C), although the size of an individual KJΔYZ cell was similar to that of an individual KJ cell, as shown by the TEM observation (Fig. 3B). Furthermore, the hy-

FIG 5 Susceptibility to human serum and neutrophils. Data represent the means from 3 independent trials. Error bars represent the standard deviations. (A) Non-immune healthy human serum sensitivity assays of the wild-type KJ and KJΔYZ strain. **, $P < 0.0001$; *, $P < 0.001$. (B) Bacterial susceptibilities to killing by human neutrophils of the wild-type KJ and KJΔYZ strains. Survival rates indicate the percentages of survival of wild-type or mutant strains calculated on the basis of viable counts relative to those for the no-neutrophil controls.

drophobicity value of KJΔYZ cells was higher than that of KJ cells (Fig. 1D). These observations support the speculation that an unidentified substance, which is a physiological substrate of the SmeYZ pump, may act as the extracellular matrix affecting the bacterial surface hydrophobicity and/or the stacking of bacterial cells but not affecting the morphology of each cell. These results

FIG 6 Virulence to mice. Mouse lethality following challenges with wild-type KJ and KJΔYZ strains is presented. Male C57BL/6 mice ($n = 9$ from the summary of two independent experiments) were inoculated by intraperitoneal injection with 1×10^9 CFU of the wild-type KJ and KJΔYZ strains. Survival was assessed for 14 days following infection.

strongly suggest that the OD₄₅₀ decline in KJΔYZ cells in the stationary phase (Fig. 1A) largely results from cell aggregation.

Cell aggregation generally correlates positively with biofilm formation. However, a contradiction occurs in KJΔYZ cells. An elevated hydrophobicity value (Fig. 1D) should confer KJΔYZ cells with a high tendency for aggregation; however, the biofilm formation of KJΔYZ cells was decreased (Fig. 4A). A possible explanation is that SmeYZ extrudes a substrate that compromises directly or indirectly the formation of the biofilm-associated extracellular matrix. This compound can be a component of such a matrix but can also be other substrates involved in biofilm formation such as quorum-sensing signals, among others.

For any infection, the prerequisite event is the encounter of the pathogenic bacteria with host cell surfaces. For this aspect, flagella can enhance the bacterial pathogenicity by providing bacterial motility (36, 37). In addition, a linkage between motility and biofilm formation has been reported (38–40). Given the fact that the *smeYZ* mutant totally abolishes flagella formation (Fig. 3B), the pleiotropic defects of mutant KJΔYZ in biofilm formation and virulence are rational.

Reactive oxygen species (ROS) are produced naturally during aerobic growth or by host immune cells during infection. Oxidative stress susceptibility is a critical determinant for bacterial survival either in physiological growth or during infection. In addition, *S. maltophilia* is frequently isolated from patients with cystic fibrosis (CF). CF lungs are known to be enriched in ROS (41, 42). In this study, we demonstrated that the *smeYZ* mutant is more susceptible to redox compounds (H₂O₂ and menadione) (Fig. 2) and human serum and neutrophils (Fig. 5). Therefore, the SmeYZ pump might play a critical role in the oxidative balance for normal physiology. Furthermore, during colonization or infection, SmeYZ can protect *S. maltophilia* from the attack of antimicrobial agents and ROS existing in human serum or neutrophils. The involvement of efflux pumps in the alleviation of oxidative stress has been reported in MacABC of *Salmonella enteric* serovar Typhimurium (43) and MacABCsm of *S. maltophilia* (30). Although the exact mechanism as to how SmeYZ alleviates oxidative stresses is not clear right now, the SmeYZ pump may export cellular components damaged by ROS or antioxidants to lower the amount of extracellular H₂O₂.

The involvement of efflux pumps in virulence has been reported in several microorganisms. The proposed mechanisms include the following: (i) efflux pumps contribute to the production of virulence factors (44, 45); (ii) efflux pumps can extrude host-derived antimicrobial molecules and alleviate the host-mediated attack (46, 47); and (iii) efflux pumps expel certain molecules, which extensively modulate bacterial physiology and thus indirectly affect virulence (48). In view of the pleiotropic phenotype change in KJΔYZ cells, aminoglycosides should not be the sole substrate for the SmeYZ pump. The contribution of SmeYZ to virulence should be attributed to extrusion of some unidentified substrates that are directly or indirectly involved in virulence. Further elucidation of the exact substrates of the SmeYZ pump and the underlying mechanisms responsible for virulence is worthwhile.

Clinical *S. maltophilia* isolates with different SmeYZ expression levels, from low to high, have been reported (21). Furthermore, the strain KJ used in this article is a clinical isolate from a patient without aminoglycoside treatment (22). These facts support the observation that SmeYZ overexpression has clinical relevance and imply that aminoglycoside extrusion seems not to be the signifi-

cant outcome of SmeYZ overexpression. The impact of efflux pump overexpression on bacterial fitness has been reported, but no consistent conclusion has been made yet. SmeDEF overexpression in *S. maltophilia* has a fitness cost (49); nevertheless, MtrCDE overexpression in *Neisseria gonorrhoeae* confers increased fitness *in vivo* (50). The actual impact of SmeYZ overexpression on fitness has not been assessed. However, we demonstrated in this article that inactivation of an overexpressed SmeYZ pump reduces motility, biofilm formation, oxidative stress susceptibility, and virulence. Accordingly, SmeYZ is an attractive target for the development of new therapeutic strategies for *S. maltophilia* infection, such as an effective inhibitor(s) against the SmeYZ pump.

ACKNOWLEDGMENT

This work was supported by grant NSC 101-2320-B-010-053-MY3 from the Ministry of Science and Technology, Taiwan.

REFERENCES

- Sanchez MB, Hernandez A, Martinez JL. 2009. *Stenotrophomonas maltophilia* drug resistance. *Future Microbiol* 4:655–660. <http://dx.doi.org/10.2217/fmb.09.45>.
- Brooke JS. 2012. *Stenotrophomonas maltophilia*: an emerging global opportunistic pathogen. *Clin Microbiol Rev* 25:2–41. <http://dx.doi.org/10.1128/CMR.00019-11>.
- Bolhuis H, van Veen HW, Poolman B, Driessen AJ, Konings WN. 1997. Mechanisms of multidrug transporters. *FEMS Microbiol Rev* 21:55–84. <http://dx.doi.org/10.1111/j.1574-6976.1997.tb00345.x>.
- Misra R, Bavro VN. 2009. Assembly and transport mechanism of tripartite drug efflux systems. *Biochim Biophys Acta* 1794:817–825. <http://dx.doi.org/10.1016/j.bbapap.2009.02.017>.
- Poole K. 2008. Bacteria multidrug efflux pumps serve other functions. *Microbe Mag* 3:179–185.
- Baugh S, Ekanayaka AS, Piddock LJV, Webber MA. 2012. Loss of or inhibition of all multidrug resistance efflux pumps of *Salmonella enterica* serovar Typhimurium results in impaired ability to form a biofilm. *J Antimicrob Chemother* 67:2409–2417. <http://dx.doi.org/10.1093/jac/dks228>.
- Chan YY, Chua KL. 2005. The *Burkholderia pseudomallei* BpeAB-OprB efflux pump: expression and impact on quorum sensing and virulence. *J Bacteriol* 187:4707–4719. <http://dx.doi.org/10.1128/JB.187.14.4707-4719.2005>.
- Pérez A, Poza M, Fernández A, Fernández M del C, Mallo S, Merino M, Rumbo-Feal S, Cabral MP, Bou G. 2012. Involvement of the AcrAB-TolC efflux pump in the resistance, fitness, and virulence of *Enterobacter cloacae*. *Antimicrob Agents Chemother* 56:2084–2090. <http://dx.doi.org/10.1128/AAC.05509-11>.
- Lister IM, Raftery C, Meccas J, Levy SB. 2012. *Yersinia pestis* AcrAB-TolC in antibiotic resistance and virulence. *Antimicrob Agents Chemother* 56:1120–1123. <http://dx.doi.org/10.1128/AAC.05338-11>.
- Chen H, Hu J, Chen PR, Lan L, Li Z, Hicks LM, Dinner AR, He C. 2008. The *Pseudomonas aeruginosa* multidrug efflux regulator MexR uses an oxidation-sensing mechanism. *Proc Natl Acad Sci U S A* 105:13586–13591. <http://dx.doi.org/10.1073/pnas.0803391105>.
- Fraud S, Poole K. 2011. Oxidative stress induction of the MexXY multidrug efflux genes and promotion of aminoglycoside resistance development in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 55:1068–1074. <http://dx.doi.org/10.1128/AAC.01495-10>.
- Palyada K, Sun YQ, Flint A, Butcher J, Naikare H, Stintzi A. 2009. Characterization of the oxidative stress stimulon and PerR regulon of *Campylobacter jejuni*. *BMC Genomics* 10:481. <http://dx.doi.org/10.1186/1471-2164-10-481>.
- Fetar H, Gilmour C, Klinoski R, Daigle DM, Dean CR, Poole K. 2011. MexEF-OprN multidrug efflux operon of *Pseudomonas aeruginosa*: regulation by the MexT activator in response to nitrosative stress and chloramphenicol. *Antimicrob Agents Chemother* 55:508–514. <http://dx.doi.org/10.1128/AAC.00830-10>.
- Fraud S, Campigotto AJ, Chen Z, Poole K. 2008. MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response sigma factor. *Antimicrob Agents Chemother* 52:4478–4482. <http://dx.doi.org/10.1128/AAC.01072-08>.

15. Crossman LC, Gould VC, Dow JM, Vernikos GS, Okazaki A, Sebahia M, Saunders D, Arrowsmith C, Carver T, Peters N, Adlem E, Kerhornou A, Lord A, Murphy L, Seeger K, Squares R, Rutter S, Quail MA, Rajandream MA, Harris D, Churcher C, Bentley SD, Parkhill J, Thomson NR, Avison MB. 2008. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Genome Biol* 9:R74. <http://dx.doi.org/10.1186/gb-2008-9-4-r74>.
16. Li XZ, Zhang L, Poole K. 2002. SmeC, an outer membrane multidrug efflux protein of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 46:333–343. <http://dx.doi.org/10.1128/AAC.46.2.333-343.2002>.
17. Alonso A, Martinez JL. 2000. Cloning and characterization of SmeDEF, a novel multidrug efflux pump from *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 44:3079–3086. <http://dx.doi.org/10.1128/AAC.44.11.3079-3086.2000>.
18. Huang YW, Liou RS, Lin YT, Huang HH, Yang TC. 2014. A linkage between SmeIJK efflux pump, cell envelope integrity, and σ^E -mediated envelope stress response in *Stenotrophomonas maltophilia*. *PLoS One* 9:e111784. <http://dx.doi.org/10.1371/journal.pone.0111784>.
19. Lin CW, Huang YW, Hu RM, Yang TC. 2014. SmeOP-TolCsm efflux pump contributes to the multidrug resistance of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 58:2405–2408. <http://dx.doi.org/10.1128/AAC.01974-13>.
20. Chen CH, Huang CC, Chung TC, Hu RM, Huang YW, Yang TC. 2011. Contribution of resistance-nodulation-division efflux pump operon *smeU1-V-W-U2-X* to multidrug resistance of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 55:5826–5833. <http://dx.doi.org/10.1128/AAC.00317-11>.
21. Gould VC, Okazaki A, Avison MB. 2013. Coordinate hyperproduction of SmeZ and SmeJK efflux pumps extends drug resistance in *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 57:655–657. <http://dx.doi.org/10.1128/AAC.01020-12>.
22. Hu RM, Huang KJ, Wu LT, Hsiao YJ, Yang TC. 2008. Induction of L1 and L2 β -lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 52:1198–1200. <http://dx.doi.org/10.1128/AAC.00682-07>.
23. Yang TC, Huang YW, Hu RM, Huang SC, Lin YT. 2009. AmpD_I is involved in expression of chromosomal L1 and L2 β -lactamases of *Stenotrophomonas maltophilia*. *Antimicrob Agents Chemother* 53:2902–2907. <http://dx.doi.org/10.1128/AAC.01513-08>.
24. Lin CW, Chiou CS, Chang YC, Yang TC. 2008. Comparison of pulsed-field gel electrophoresis and three rep-PCR methods for evaluating the genetic relatedness of *Stenotrophomonas maltophilia* isolates. *Lett Appl Microbiol* 47:393–398. <http://dx.doi.org/10.1111/j.1472-765X.2008.02443.x>.
25. Clinical and Laboratory Standards Institute. 2012. Performance standards for antimicrobial susceptibility testing; 22nd informational supplement. CLSI document M100-S22. Clinical and Laboratory Standards Institute, Wayne, PA.
26. Zhao X, Wang Y, Shang Q, Li Y, Hao H, Zhang Y, Guo Z, Yang G, Xie Z, Wang R. 2015. Collagen-like proteins (ClpA, ClpB, ClpC, and ClpD) are required for biofilm formation and adhesion to plant roots by *Bacillus amyloliquefaciens* FZB42. *PLoS One* 10:e0117414. <http://dx.doi.org/10.1371/journal.pone.0117414>.
27. Hoopman TC, Liu W, Joslin SN, Pybus C, Brautigam CA, Hansen EJ. 2011. Identification of gene products involved in the oxidative stress response of *Moraxella catarrhalis*. *Infect Immun* 79:745–755. <http://dx.doi.org/10.1128/IAI.01060-10>.
28. Murray TS, Kazmierczak BI. 2006. FlhF is required for swimming and swarming in *Pseudomonas aeruginosa*. *J Bacteriol* 188:6995–7004. <http://dx.doi.org/10.1128/JB.00790-06>.
29. Kirov SM, Tassell BC, Semmler ABT, O'Donovan LA, Rabaan AA, Shaw JG. 2002. Lateral flagella and swarming motility in *Aeromonas* species. *J Bacteriol* 184:547–555. <http://dx.doi.org/10.1128/JB.184.2.547-555.2002>.
30. Lin YT, Huang YW, Liou RS, Yang TC. 2014. MacABCsm, an ABC-type tripartite efflux pump of *Stenotrophomonas maltophilia*, involved in drug resistance, oxidative and envelope stress tolerance, and biofilm formation. *J Antimicrob Chemother* 69:3221–3226. <http://dx.doi.org/10.1093/jac/dku317>.
31. Kumar L, Chhibber S, Harjai K. 2014. Structural alterations in *Pseudomonas aeruginosa* by zingerone contribute to enhanced susceptibility to antibiotics, serum and phagocytes. *Life Sci* 117:24–32. <http://dx.doi.org/10.1016/j.lfs.2014.09.017>.
32. Dacheux D, Attree I, Schneider C, Toussaint B. 1999. Cell death of human polymorphonuclear neutrophils induced by a *Pseudomonas aeruginosa* cystic fibrosis isolate requires a functional type III secretion system. *Infect Immun* 67:6164–6167.
33. Pan CY, Chen JC, Sheen JF, Lin TL, Chen JY. 2014. Epinecidin-1 has immunomodulatory effects, facilitating its therapeutic use in a mouse model of *Pseudomonas aeruginosa* sepsis. *Antimicrob Agents Chemother* 58:4264–4274. <http://dx.doi.org/10.1128/AAC.02958-14>.
34. Toleman MA, Bennett PM, Bennett DM, Jones RN, Walsh TR. 2007. Global emergence of trimethoprim/sulfamethoxazole resistance in *Stenotrophomonas maltophilia* mediated by acquisition of *sul* genes. *Emerg Infect Dis* 13:559–565. <http://dx.doi.org/10.3201/eid1304.061378>.
35. Huang YW, Hu RM, Lin YT, Huang HH, Yang TC. 2015. The contribution of class I integron to antimicrobial resistance in *Stenotrophomonas maltophilia*. *Microb Drug Resist* 21:90–96. <http://dx.doi.org/10.1089/mdr.2014.0072>.
36. Josenhans C, Suerbaum S. 2002. The role of motility as a virulence factor in bacteria. *Int J Med Microbiol* 291:605–614. <http://dx.doi.org/10.1078/1438-4221-00173>.
37. Duan Q, Zhou M, Zhu L, Zhu G. 2013. Flagella and bacterial pathogenicity. *J Basic Microbiol* 53:1–8. <http://dx.doi.org/10.1002/jobm.201100335>.
38. Wood TK, Gonzalez AF, Herzberg M, Lee J. 2006. Motility influences biofilm architecture in *Escherichia coli*. *Appl Microbiol Biotechnol* 72:361–367. <http://dx.doi.org/10.1007/s00253-005-0263-8>.
39. Lemon KP, Higgins DE, Kolter R. 2007. Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *J Bacteriol* 189:4418–4424. <http://dx.doi.org/10.1128/JB.01967-06>.
40. Kim TJ, Young BM, Young GM. 2008. Effect of flagellar mutations on *Yersinia enterocolitica* biofilm formation. *Appl Environ Microbiol* 74:5466–5474. <http://dx.doi.org/10.1128/AEM.00222-08>.
41. Vaisman N, Kerasin E, Hahn T, Trifon S, Voet H, Tabachnik E. 1994. Increased neutrophil chemiluminescence production in patients with cystic fibrosis. *Metabolism* 43:719–722. [http://dx.doi.org/10.1016/0026-0495\(94\)90120-1](http://dx.doi.org/10.1016/0026-0495(94)90120-1).
42. Ciofu O, Riis B, Pressler T, Poulsen HE, Hoiby N. 2005. Occurrence of hypermutable *Pseudomonas aeruginosa* in cystic fibrosis patients is associated with the oxidative stress caused by chronic lung inflammation. *Antimicrob Agents Chemother* 49:2276–2282. <http://dx.doi.org/10.1128/AAC.49.6.2276-2282.2005>.
43. Bogomolnaya LM, Andrews KD, Talamantes M, Maple A, Ragoza Y, Vazquez-Torres A, Andrews-Polymenis H. 2013. The ABC-type efflux pump MacAB protects *Salmonella enterica* serovar Typhimurium from oxidative stress. *mBio* 4:e00630–13. <http://dx.doi.org/10.1128/mBio.00630-13>.
44. Bina XR, Provenzano D, Nguyen N, Bina JE. 2008. *Vibrio cholerae* RND family efflux pump systems are required for antimicrobial resistance, optimal virulence factor production, and colonization of the infant mouse small intestine. *Infect Immun* 76:3595–3605. <http://dx.doi.org/10.1128/IAI.01620-07>.
45. Taylor DL, Bina XR, Bina JE. 2012. *Vibrio cholerae* VexH encodes a multiple drug efflux pump that contributes to the production of cholera toxin and the toxin co-regulated pilus. *PLoS One* 7:e38208. <http://dx.doi.org/10.1371/journal.pone.0038208>.
46. Coudeyras S, Nakusi L, Charbonnel N, Forestier C. 2008. A tripartite efflux pump involved in gastrointestinal colonization by *Klebsiella pneumoniae* confers a tolerance response to inorganic acid. *Infect Immun* 76:4633–4641. <http://dx.doi.org/10.1128/IAI.00356-08>.
47. Pletzer D, Weingart H. 2014. Characterization and regulation of the resistance-nodulation-cell division-type multidrug efflux pumps MdtABC and MdtUVW from the fire blight pathogen *Erwinia amylovora*. *BMC Microbiol* 14:185. <http://dx.doi.org/10.1186/1471-2180-14-185>.
48. Lamarche MG, Deziel E. 2011. MexEF-OprN efflux pump exports the *Pseudomonas* quinolone signal (PQS) precursor HHQ (4-hydroxy-2-heptylquinoline). *PLoS One* 6:e24310. <http://dx.doi.org/10.1371/journal.pone.0024310>.
49. Alonso A, Morales G, Escalante R, Campanario E, Sastre L, Martinez JL. 2004. Overexpression of the multidrug efflux pump SmeDEF impairs *Stenotrophomonas maltophilia* physiology. *J Antimicrob Chemother* 53:432–434. <http://dx.doi.org/10.1093/jac/dkh074>.
50. Warner DM, Shafer WM, Jerse AE. 2008. Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and *in vivo* fitness. *Mol Microbiol* 70:462–478. <http://dx.doi.org/10.1111/j.1365-2958.2008.06424.x>.