The role of the *Serratia marcescens* SdeAB multidrug efflux pump and TolC homologue in fluoroquinolone resistance studied via gene-knockout mutagenesis

Sanela Begic and Elizabeth A. Worobec

Department of Microbiology, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

Correspondence

Elizabeth A. Worobec

eworobe@cc.umanitoba.ca

Serratia marcescens is a prominent opportunistic nosocomial pathogen resistant to several classes of antibiotics. The major mechanism for fluoroquinolone resistance in various Gram-negative pathogens is active efflux. Our group previously identified SdeAB, a resistance-nodulation-cell division (RND) efflux pump complex, and a TolC-like outer-membrane protein (HasF), which together mediate energy-dependent fluoroquinolone efflux. In addition, a regulatory protein-encoding gene in the upstream region of *sdeAB* was identified (*sdeR*) and found to be 40 % homologous to MarA, an *Escherichia coli* transcriptional regulator. To provide conclusive evidence as to the role of these components in *S. marcescens*, *sdeB*, *hasF* and *sdeR* deletion mutants were constructed. Suicide vectors were created and introduced via triparental mating into *S. marcescens* UOC-67 (wild-type) and, for *sdeB* and *hasF*, T-861 (clinical isolate). We have analysed these genetically altered strains using minimal inhibitory concentration (MIC) assays for a wide range of compounds (fluoroquinolones, SDS, novobiocin, ethidium bromide and chloramphenicol). Intracellular accumulation of a variety of fluoroquinolones was measured fluorospectroscopically. The *sdeB*, *hasF* and *sdeR* knockout strains were consistently more susceptible to antibiotics than the parent strains, with the *sdeB/hasF* double knockout strain showing the highest susceptibility. A marked increase in fluoroquinolone (ciprofloxacin) accumulation was observed for strains deficient in either the *sdeB* or *hasF* genes when compared to the parental strains, with the highest ciprofloxacin accumulation observed for the *sdeB/hasF* double knockout. Antibiotic accumulation assays for the *sdeB* knockout mutant strains performed in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a proton-motive-force inhibitor, demonstrated that SdeAB-mediated efflux is proton-motive-force dependent. Due to the comparable susceptibility of the *sdeB* and the *hasF* individual knockouts, we conclude that *S. marcescens* HasF is the sole outer-membrane component of the SdeAB pump. In addition, MIC data for *sdeR*-deficient and overexpressing strains confirm that SdeR is an activator of *sdeAB* and acts to enhance the overall multidrug resistance of *S. marcescens*.

Received 13 August 2007

Revised 22 October 2007

Accepted 12 November 2007

INTRODUCTION

Serratia marcescens is a prominent opportunistic pathogen, responsible for serious infections in immunocompromised individuals due primarily to its high intrinsic antibiotic resistance. It has been shown to be resistant to all major classes of antibiotics used, including fluoroquinolones, making the treatment of infections very difficult (Lambert & O'Grady, 1992; Aucken & Pitt, 1998; Fujimaki *et al.*, 1989). The major mechanism for fluoroquinolone resistance in various Gram-negative organisms, including *S. marcescens*, is the active efflux of the antibiotic molecule mediated

by efflux pumps belonging to the resistance-nodulation-cell division (RND) family. RND pumps work in conjunction with a periplasmic protein and an outer-membrane protein (e.g. *Escherichia coli* TolC) to form a tripartite system (Li & Nikaido, 2004, and references therein).

We have established active efflux as a resistance mechanism in *S. marcescens*, and presented molecular characterization of two different efflux pumps (Kumar & Worobec, 2005a). Of these, we have demonstrated that the SdeAB RND pump is a multidrug efflux pump with a wide range of substrates, having a high degree of homology to the AcrAB pump of *E. coli*. The *sdeAB* operon consists of the *sdeA* gene, encoding a periplasmic fusion protein, and the *sdeB* gene, encoding the transporter of the RND pump on the

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; RND, resistance-nodulation-cell division.

inner membrane. We have also reported on an outer-membrane component called HasF (TolC homologue) involved in energy-dependent efflux of antimicrobial agents (Kumar & Worobec, 2005b). Computer-generated analysis of the *S. marcescens* HasF revealed a very similar structure to that of *E. coli* TolC, having the channel-tunnel structure characteristic of outer-membrane components of RND efflux pumps. No other *tolC* homologue was found upon searching the *S. marcescens* genome (Kumar & Worobec, 2005b; http://www.sanger.ac.uk/Projects/S_marcescens/).

Most of the efflux pumps characterized to date have a regulatory protein-encoding gene upstream from the pump-encoding genes (Aleksun & Levy, 1997; Barbosa & Levy, 2000; Hachler *et al.*, 1991). Upstream from the *sdeAB* locus is *sdeR*, a 405 bp ORF which is transcribed in the opposite direction to *sdeAB* (Kumar, 2004). At the amino acid level, SdeR is 40 % homologous to the MarA protein of *E. coli*, a transcriptional activator of the AcrAB-TolC drug efflux pump (Aleksun & Levy, 1997; Barbosa & Levy, 2000; Hachler *et al.*, 1991), and *sdeR* is 50 % homologous to *marA* at the DNA level. Amino acid prediction and three-dimensional structural prediction also showed similarity to the MarA protein of *E. coli*, with a high degree of conservation of the DNA-binding helices (results not shown).

In this study, we addressed the importance of the SdeAB pump and the TolC-like protein in fluoroquinolone resistance by constructing *hasF* and *sdeB* gene knockouts. In addition, through knockout mutagenesis, we have demonstrated the importance of SdeR in the regulation of the expression of the SdeAB efflux pump system.

METHODS

Bacterial strains and plasmids. All strains and plasmids used in this study are outlined in Table 1. All bacterial strains were grown in Luria-Bertani agar (LB), Trypticase Soy Agar (TSA) and Tryptone Soy Broth (TSB) (BD Diagnostics Systems). The ampicillin-resistant strains (containing pKS(+), pKS:SdeB, pKS:HasF, pKS:ΔHasF, pKS:SdeR) were grown on LB plates and in LB broth containing 100 µg ampicillin ml⁻¹ (Sigma-Aldrich). The kanamycin-resistant strains (with pKIXX, pKS:SdeR:Km^r, pKS:SdeB:Km^r) were grown on LB plates and in LB broth containing 25 µg kanamycin ml⁻¹ (Sigma-Aldrich). The streptomycin-resistant strains (with pKNG101, pKNGsdeB, pKNGhasF, pKNGsdeR) were grown on LB plates and in LB broth containing 50 µg streptomycin ml⁻¹ (Sigma-Aldrich). The carbenicillin-resistant strains (with pEX1.8, pEXSH, pEXH, pEXS and pEXR) were grown on LB plates and in LB broth containing 300 µg carbenicillin ml⁻¹ (Sigma-Aldrich). Strains SDEAB1, SDEAB2, HASF100, HASF200, SDEAB3/HASF300 and SDER1 were grown on LB plates and in LB broth containing 100 µg ampicillin ml⁻¹ and 50 µg streptomycin ml⁻¹. UOC-67, MT616, CC118 and T-861 were grown in the absence of any antibiotic.

Construction of *S. marcescens* *hasF*-, *sdeB*- and *sdeR*-deficient strains. Unique *HincII/KpnI* restriction sites within pKS:HasF (Kumar & Worobec, 2005b) separated by approximately 700 bases were identified to be a feasible target for creating a deletion mutation in the *hasF* coding region. After transformation into *S. marcescens* UOC-67, the mutant clone was confirmed by restriction digestion, PCR and sequencing. The altered *hasF* fragment was then transferred into the

pKNG101 replacement vector and cloned into *E. coli* CC118 for stability. pKNG101 is a suicide vector that contains a conditional origin of replication (*oriR6K*), the *strAB* genes encoding streptomycin phosphotransferase (Str^r), an origin of transfer (*mobRK2*), the *sacB* gene mediating sucrose sensitivity, and multiple cloning sites (Kaniga *et al.*, 1991). The *sdeB* gene (Kumar & Worobec, 2005a) was recloned as a 1.7 kb *EcoRI/BglII* fragment into pKS, and transformed into UOC-67 in order to be disrupted by the insertion of a kanamycin-resistance cassette into a unique *PstI* restriction site. The *sdeR* gene (GenBank accession no. AY623133) was recloned as a 0.4 kb *BamHI-EcoRI* fragment into pKS, and transformed into UOC-67 in order to be disrupted by the insertion of a kanamycin-resistance cassette into a unique *EcoRV* restriction site. The disrupted *sdeB* and *sdeR* fragments were transferred into the pKNG101 replacement vector and cloned into *E. coli* CC118 in a similar fashion as for *hasF*. Conjugation between the *E. coli* strain harbouring each of the disrupted *S. marcescens* *sdeB*, *hasF* and *sdeR* genes and the wild-type *S. marcescens* strain was carried out using *E. coli* helper strain MT616 (Finan *et al.*, 1986) to create SDEAB1, HASF100 and SDER1, respectively. Conjugation was also carried out between the *E. coli* strain harbouring each of the disrupted *S. marcescens* *sdeB* and *hasF* genes and the *S. marcescens* clinical isolate strain (T-861), using *E. coli* helper strain MT616 (Finan *et al.*, 1986) for comparison purposes. For the double *sdeB/hasF*-deficient mutant (SDEAB3/HASF300), conjugation was carried out between the *S. marcescens* *sdeB* (SDEAB1)- and *hasF* (HASF100)-deficient strains, using *E. coli* MT616 as a helper. Analysis of transconjugants was based on sucrose sensitivity due to the synthesis of lethal levano compounds, catalysed by levansucrase, the product of the *sacB* gene. All mutations were confirmed by restriction digestion, sequence analysis of both strands (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada) and SDS-PAGE protein analysis.

Complementation of *sdeB*-, *hasF*- and *sdeR*-deficient strains.

For complementation purposes, the expression vector pEX1.8 was used for cloning a 0.7 kb wild-type *HincII/KpnI* *hasF* fragment (pEXH), a 1.7 kb wild-type *EcoRI/BglII* *sdeB* fragment (pEXS) and a 0.4 kb wild-type *BamHI/EcoRI* *sdeR* fragment (pEXR). In addition, *hasF* and *sdeB* were introduced into SDEAB3/HASF300 (*sdeB/hasF*-deficient strain). To complement both *hasF* and *sdeB* simultaneously, the 1.7 kb *EcoRI/BglII* *sdeB* fragment was cloned into the *EcoRI/BglII* site, and the 0.7 kb *hasF* *HincII/KpnI* fragment was cloned into the *HincII/KpnI* site of pEX1.8 (pEXSH). To construct strain SM2000, pEXH was first transferred into the UOC-67 by electroporation. Conjugation between UOC-67/pEXH harbouring the wild-type *hasF* gene and SDEAB3/HASF300 (*sdeB/hasF*-deficient) was carried out using the *E. coli* helper strain. The same two-step procedure was used to transform pEXS and pEXSH into SDEAB3/HASF300 and pEXR into SDER1 to create SM3000, SM1000 and SDER2, respectively. Selection of transconjugants was achieved by incorporation of ampicillin and streptomycin into the growth medium. Transformants were verified by restriction digestion and sequence analysis of both strands of the wild-type genes (National Research Council, Plant Biotechnology Institute, Saskatoon, Canada). Proteins were expressed under the control of the *P_{tac}* promoter on pEX1.8 and induced by the addition of varying concentrations (0.5–5 mmol l⁻¹) of IPTG (Sigma-Aldrich) to the growth medium.

***sdeR* overexpression.** A 0.4 kb *BamHI-EcoRI* *sdeR* PCR fragment (including the entire open reading frame and ribosome-binding site) was cloned into the *BamHI/EcoRI* site of pEX1.8 (pEXR) and transformed into UOC-67 to generate SDER3. pEX1.8 alone was also transformed into UOC-67 to generate SDER4 to use as a control. Transformants were confirmed via restriction digestion and sequence analysis.

Antibiotic susceptibility tests. Susceptibility of SDEAB1, SDEAB2, HASF100, HASF200, SDEAB3/HASF300, SM1000, SM2000, SM3000, SDER1, SDER2, SDER3 and SDER4 to norfloxacin, ciprofloxacin,

Table 1. Bacterial strains and plasmids used in the study

Abbreviations: Kan, kanamycin; Amp, ampicillin; Str, streptomycin; Car, carbenicillin.

Strain or plasmid	Characteristic	Source/reference
<i>E. coli</i>		
MT616	MT607 (<i>pro-82 thi-1 hsdR17 supE44</i>):pRK600	Finan <i>et al.</i> (1986)
CC118	<i>araD139 Δ(ara leu)7697 ΔlacX74 phoA20 galE galK thi rpsE rpoB argEam recA1</i>	C. Manoil, University of Washington, Seattle, USA
<i>S. marcescens</i>		
UOC-67	Wild-type, ATCC 13880	American Type Culture Collection
T-861	Clinical isolate	D. Hoban, Health Science Centre, Winnipeg
SDEAB1	<i>sdeB</i> -deficient UOC-67	This study
SDEAB2	<i>sdeB</i> -deficient T-861	This study
HASF100	<i>hasF</i> -deficient UOC-67	This study
HASF200	<i>hasF</i> -deficient T-861	This study
SDEAB3/HASF300	<i>sdeB/hasF</i> -deficient UOC-67	This study
SDER1	<i>sdeR</i> -deficient UOC-67	This study
SDER2	SDER1/pEXR	This study
SM3000	SDEAB3/HASF300 with pEXS	This study
SM2000	SDEAB3/HASF300 with pEXH	This study
SM1000	SDEAB3/HASF300 with pEXSH	This study
SDER3	UOC-67/pEXR	This study
SDER4	UOC-67/pEX1.8	This study
Plasmids		
pKS (+)	ColE1 replicon, Amp ^r , <i>lacZ'</i>	Stratagene
pKS:SdeB	pKS with 1.7 kb <i>EcoRI/BglI sdeB</i> fragment	This study
pKS:SdeB:Km ^r	pKS:SdeB with 1.4 kb Kan ^r insertion in <i>sdeB</i>	This study
pKS:HasF	pKS with 1.5 kb <i>EcoRI/BamHI hasF</i> fragment	This study
pKS:ΔHasF	pKS:HasF with <i>HincII/KpnI</i> 700 bp deleted	This study
pKS:SdeR	pKS with 400-base <i>BamHI/EcoRI sdeR</i> fragment	This study
pKS:SdeR:Km ^r	pKS:SdeR with a 1.4 kb Kan ^r insertion in <i>sdeR</i>	This study
pEX1.8	CoE1 replicon, Amp ^r Car ^r , carries <i>lacI^Q</i> and a <i>P_{tac}</i> promoter	Pearson <i>et al.</i> (1997)
pEXSH	pEX1.8 with wild-type 700 bp <i>hasF</i> and 1.7 kb <i>sdeB</i> fragments	This study
pEXH	pEX1.8 with wild-type 700 bp <i>hasF</i> fragment	This study
pEXS	pEX1.8 with wild-type 1.7 kb <i>sdeB</i> fragment	This study
pEXR	pEX1.8 with 405 bp <i>BamHI/EcoRI sdeR</i> fragment	This study
pKNG101	Suicide vector, <i>pir⁻ oriR6K mobRK2 sacB Str^r</i>	Kaniga <i>et al.</i> (1991)
pKNGsdeB	pKNG101 with 3.1 kb insertionally disrupted <i>sdeB</i> fragment	This study
pKNGhasF	pKNG101 with 800 bp deleted <i>hasF</i>	This study
pKNGsdeR	pKNG101 with 1.8 kb insertionally disrupted <i>sdeR</i> fragment	This study
pKIXX	1.4 kb Kan ^r cassette flanked by <i>SmaI</i>	Pharmacia PL Biochemicals

ofloxacin, chloramphenicol, novobiocin, SDS and ethidium bromide (Sigma-Aldrich) was tested using the minimum inhibitory concentration (MIC) twofold broth dilution method (CLSI, 2006). Overnight cultures in TSB were diluted 1000-fold in fresh broth, grown at 37 °C to OD₆₀₀ 0.5–0.9 and 5 µl of the bacterial suspension was inoculated in TSB containing serial dilutions of each antibiotic. Results are reported as MIC, the concentration of antibiotic that inhibited visible growth determined by absence of turbidity in TSB after 18 h shaking incubation at 37 °C.

Fluoroquinolone accumulation. The accumulation of ciprofloxacin by SDEAB1, SDEAB2, HASF100, HASF200, SDEAB3/HASF300, SM1000, SM2000 and SM3000 was measured using the method of Mortimer & Piddock (1991). Cultures were grown until the OD₆₀₀ reached 0.5–0.7. Cells were harvested by centrifugation at 4000 g for 15 min at room temperature, suspended in PBS pH 7.5 and washed twice. Pellets were suspended in 1/10 volume of PBS. Ciprofloxacin

was added to 1 ml aliquots to reach 10 µg ml⁻¹ external concentration and allowed to incubate at room temperature for 1–12 min. Carbonyl cyanide *m*-chlorophenylhydrazon (CCCP) (Sigma-Aldrich) was added to a final concentration of 100 µM after 5 min antibiotic incubation. After incubation, a rapid centrifugation (13 000 g at 4 °C for 1 min) was carried out, followed by a wash in cold PBS, suspension in 1 ml 0.1 M glycine.HCl pH 3.0 to lyse the cells, and overnight incubation at room temperature. This suspension was centrifuged at 11 000 g for 5 min to remove cellular debris and antibiotic concentration measured in a Shimadzu RF-1501 spectrofluorometer. The fluorescence of ciprofloxacin was measured at 279 nm excitation wavelength and 447 nm emission wavelength. Ciprofloxacin concentration was calculated using a standard curve (concentration ranging from 100 to 1000 ng) in 0.1 M glycine.HCl pH 3.0. The results were expressed as nanograms of ciprofloxacin incorporated per milligram (dry weight) of bacteria. Results in the figures are means and standard errors.

RESULTS

Antibiotic susceptibility tests

The most dramatic increase in antibiotic susceptibility was found with the double-knockout strain (SDEAB3/HASF300) in comparison to UOC-67 (Table 2). For SDEAB1 and HASF100, susceptibility to all compounds tested increased in comparison to UOC-67, and for SDEAB2 and HASF200 in comparison to T-861. MIC values for HASF100 were identical to those of SDEAB1 for all compounds tested, except for chloramphenicol, where there was a slight difference. A similar trend was found for HASF200 and SDEAB2. MIC values for SM1000 (*hasF/sdeB*-complemented strain), SM2000 (*hasF*-complemented strain) and SM3000 (*sde*-complemented strain) were identical to those of wild-type UOC-67 for all antibiotics tested, suggesting that both SdeB and HasF are equally important for *S. marcescens* multidrug resistance.

The *sdeR*-overexpressing strain (SDER3) was considerably more resistant than either UOC-67 or SDER4 (UOC-67/pEX1.8) (Table 2) for all compounds tested, while SDER1 (*sdeR*-deficient) was more susceptible to all compounds. Complementation of SDER1 with *sdeR* (SDER2) resulted in a restoration of resistance to wild-type levels.

Fluoroquinolone accumulation

The loss of the functioning efflux pump SdeAB (SDEAB1 and SDEAB2) resulted in a significant increase in ciprofloxacin accumulation as compared to UOC-67 (Fig. 1a) and T-861 parental strains, respectively (Fig. 1b).

The addition of CCCP, a proton-motive-force inhibitor, increased the accumulation rate of UOC-67 (Fig. 1a) and T-861 (Fig. 1b); however, it had no effect upon ciprofloxacin accumulation in the knockout mutant strains. Ciprofloxacin accumulation for the SM3000 (*sdeB*-complemented) strain was very similar to that of the UOC-67 in the absence and presence of CCCP.

The loss of the functioning *hasF* gene (HASF100 and HASF200) also resulted in a notable increase in ciprofloxacin accumulation as compared to UOC-67 (Fig. 2a) and T-861 parental strains, respectively (Fig. 2b). As expected, the addition of CCCP increased the accumulation of UOC-67 (Fig. 2a) and T-861 (Fig. 2b); however, it had no effect upon ciprofloxacin accumulation in the HASF100 and HASF200 knockout mutant strains. Ciprofloxacin accumulation for the SM2000 (*hasF* complemented) strain was very similar to that of the UOC-67 in the absence and presence of CCCP.

Although the accumulation of SDEAB1 and HASF100 was almost identical, the loss of both *hasF* and *sdeB* (SDEAB3/HASF300) resulted in increased ciprofloxacin accumulation (a 30 % increase in SDEAB3/HASF100 as compared to SDEAB1 or HASF100 alone) (Fig. 3). Addition of CCCP resulted in a further increase in ciprofloxacin accumulation for the double knockout strain (SDEAB3/HASF300) resulting in a 40 % increase in accumulation as compared to HASF100 in the absence of CCCP (Fig. 3). SM1000 (*sdeB/hasF*-complemented) in the absence of CCCP showed the lowest accumulation, lower than that of either SDEAB1 or HASF100 alone, suggesting that *sdeB* and *hasF* have a synergistic effect. In the presence of CCCP, SM1000 showed a significant increase in accumulation.

Table 2. Susceptibilities of wild-type, clinical isolate and various *sdeB* and *hasF* mutant *S. marcescens* strains to a range of antibiotics

Abbreviations: Cip, ciprofloxacin; Nor, norfloxacin; Ofi, ofloxacin; Chl, chloramphenicol; Nov, novobiocin; SDS, sodium dodecyl sulphate; EtBr, ethidium bromide. Results are representative of three trials.

Strain	MIC ($\mu\text{g ml}^{-1}$)						
	Cip	Nor	Ofi	Chl	Nov	SDS	EtBr
UOC-67 (wild-type)	1	1	1	32	64	1	1
T-861 (clinical isolate)	128	128	128	256	256	4	8
SDEAB1 (<i>sdeB</i> -deficient in UOC-67)	0.125	0.125	0.125	4	16	0.25	0.25
SDEAB2 (<i>sdeB</i> -deficient in T-861)	0.008	0.008	0.008	16	32	0.25	0.25
HASF100 (<i>hasF</i> -deficient in UOC-67)	0.125	0.125	0.125	8	16	0.25	0.25
HASF200 (<i>hasF</i> -deficient in T-861)	0.008	0.008	0.008	32	32	0.25	0.25
SDEAB3/HASF300 (<i>sdeB/hasF</i> -deficient in UOC-67)	0.002	0.002	0.002	0.004	0.016	0.004	0.004
SM3000 (<i>sdeB</i> -complemented SDEAB1)	1	1	1	32	64	1	1
SM2000 (<i>hasF</i> -complemented HASF100)	1	1	1	32	64	1	1
SM1000 (<i>sdeB, hasF</i> -complemented SDEAB3/HASF300)	1	1	1	32	64	1	1
SDER1 (<i>sdeR</i> -deficient UOC-67)	0.25	0.25	0.25	8	8	0.25	0.25
SDER2 (<i>sdeR</i> -complemented SDER1)	1	1	1	32	64	1	1
SDER3 (<i>sdeR</i> -overexpressing UOC-67)	256	256	128	256	512	64	64
SDER4 (UOC-67 with vector)	1	1	1	32	64	1	1

Fig. 1. Accumulation of ciprofloxacin by (a) wild-type, mutant and complemented *sdeB* *S. marcescens* UOC-67 and (b) clinical isolate and mutant *sdeB* *S. marcescens* T-861 in the absence and presence of CCCP. Various strains are depicted as follows: (a) UOC-67 in presence (■) and absence (□) of CCCP; SDEAB1 in presence (▲) and absence (△) of CCCP; SM3000 in presence (●) and absence (○) of CCCP; (b) T-861 in presence (■) and absence (□) of CCCP; SDEAB2 in presence (▲) and absence (△) of CCCP. The proton-motive-force uncoupler CCCP (100 μM) was added after 5 min incubation (arrow). All trials were performed in triplicate.

Fig. 2. Accumulation of ciprofloxacin by (a) wild-type, mutant and complemented *hasF* *S. marcescens* UOC-67 and (b) clinical isolate and mutant *hasF* *S. marcescens* T-861 in the absence and presence of CCCP. Various strains are depicted as follows: (a) UOC-67 in presence (■) and absence (□) of CCCP; HASF100 in presence (▲) and absence (△) of CCCP; SM2000 in presence (●) and absence (○) of CCCP; (b) T-861 in presence (■) and absence (□) of CCCP; HASF200 in presence (▲) and absence (△) of CCCP. The proton-motive-force uncoupler CCCP (100 μM) was added after 5 min incubation (arrow). All trials were performed in triplicate.

Fig. 3. Accumulation of ciprofloxacin by *sdeB*-deficient, *hasF*-deficient, double-deficient and complemented *S. marcescens* strains. SDEAB3/HASF300 accumulation in absence and presence of CCCP is shown, whereas the CCCP results for SDEAB1 and HASF100 can be found in Figs 1 and 2. Various strains are depicted as follows: SDEAB1, no CCCP (■); HASF100, no CCCP (□); SDEAB3/HASF300, in absence (△) and presence (▲) of CCCP; SM1000, in absence (○) and presence (●) of CCCP. The proton-motive-force uncoupler CCCP (100 μ M) was added after 5 min incubation (arrow). All trials were performed in triplicate.

DISCUSSION

Three different RND pumps have been identified in *S. marcescens*: SdeAB, SdeCDE (Kumar & Worobec, 2005a) and SdeXY (Chen *et al.*, 2003). SdeCDE appears to be a homologue of the *E. coli* MdtABC system (Kumar & Worobec, 2005a). Preliminary results thus far demonstrate that SdeCDE is a very selective pump and provides very limited drug resistance, with novobiocin being its only substrate (data not shown). Novobiocin is an inhibitor of DNA gyrase that targets the GyrB subunit (Maxwell & Lawson, 2003). Similar results have been reported for MdtABC of *E. coli* (Nagakubo *et al.*, 2002). SdeXY confers norfloxacin and tetracycline resistance and appears to be a close homologue of the *E. coli* AcrAB-TolC pump (Chen *et al.*, 2003). Our study demonstrates that *S. marcescens* SdeAB is the major pump, functioning with broad substrate specificity.

sdeB and *hasF* knockouts resulted in an increase in susceptibility for all compounds tested, demonstrating that both *hasF* and *sdeB* mutants were hypersusceptible to a range of antibiotics, dyes and detergents. This finding is consistent with Buckley *et al.* (2006), where the authors reported a similar antibiotic-susceptibility trend for *acrB* and *tolC* knockouts in *Salmonella enterica*, and with Ruzin *et al.* (2007), where the same finding was reported for an *adeB* knockout strain (inner-membrane transporter of the AdeABC multidrug efflux pump homologous to AcrAB of *E. coli*). Hypersusceptibility to a variety of compounds was also reported for *acrAB* deletion mutants in *E. coli* (Ma *et al.*, 1995); AcrAB is a pump to which *S. marcescens* SdeAB is closely related. Similarly, in *Helicobacter pylori*, knockout mutant strains in which TolC homologues were inactivated displayed higher susceptibility to antibiotics and ethidium bromide (van Amsterdam *et al.*, 2005), similar to the *hasF* knockout strains. Also, in *Brucella suis*, mutations in *bepC*, encoding a TolC homologue, increased the antimicrobial susceptibility, contributing to the intrinsic resistance/susceptibility phenotype (Posadas *et al.*, 2007). Corresponding mutant strains complemented with *sdeB* or *hasF*, or both, resulted in a restoration of

resistance to wild-type levels, demonstrating the overall importance of both these components.

sdeR knockout resulted in a decrease in resistance that was restored to wild-type levels upon complementation, while overexpression of *sdeR* significantly increased resistance. These results demonstrate the role of SdeR as an activator of SdeAB expression. Similar results were found in *Enterobacter aerogenes*, where the RamA activator was shown to induce the expression of the efflux pump and act as an activator (Chollet *et al.*, 2004). SdeR is 40% homologous to the MarA protein of *E. coli*, a transcriptional activator of the AcrAB-TolC drug efflux pump, and the amino acid sequences of the two DNA-binding motifs essential to the regulatory function of MarA are well conserved in SdeR. In addition to suggesting that SdeR and MarA may recognize the same set of operator sequences, we have detected the presence of a putative *mar* box in the *sdeR* promoter that is well conserved according to the consensus (Kumar, 2004).

Strains in the study were examined spectrofluorimetrically for efflux of ciprofloxacin and other fluoroquinolones not reported (norfloxacin, ofloxacin, levofloxacin), before and after the addition of the uncoupler CCCP. Knockout mutant strains (SDEAB1, SDEAB2, HASF100, HASF200, SDEAB3/HASF300) that do not efflux antibiotics were not affected by the addition of CCCP. When the HasF outer-membrane protein (HASF100, HASF200) or both HasF and the pump (SDEAB3/HASF300) were no longer produced, mutant strains continued to accumulate antibiotics in a steady fashion before and after the addition of the uncoupler (Figs 1, 2 and 3). In contrast, antibiotic accumulation of the wild-type strain UOC-67 and the clinical T-861 isolate was low, but increased after the addition of CCCP as the proton gradient-dependent efflux collapsed (Figs 1, 2 and 3). This is a similar finding to that reported by Pumbwe & Piddock (2002), where the *cmeB* gene encoding a *Campylobacter jejuni* multidrug efflux pump was knocked out and mutant strains displayed higher ciprofloxacin accumulation than the wild-type *C. jejuni*. For strains SM3000 and SM2000, complemented

with *sdeB* and *hasF* genes, respectively, the accumulation trend was similar to that of UOC-67 where the levels increased after CCCP collapsed the gradient. This suggests that both components, SdeB and HasF, are important in the drug efflux as the accumulation levels for each are very similar to that of UOC-67. For the *sdeB/hasF* double-complemented strain SM1000, in the absence of CCCP, accumulation levels were lower than those of the corresponding uncomplemented mutants SDEAB1 or HASF100, suggesting that most of the compound is effluxed out of the cell through the restored outer-membrane channel. With the addition of CCCP, SM1000 accumulation levels increased to that of SDEAB3/HASF300 in the absence of CCCP, suggesting that the collapse of the gradient did restore the accumulation once again (Fig. 3). SM100 accumulation levels, however, were similar in trend to UOC-67 (results not shown).

Our early studies show that the SdeCDE pump does not efflux ciprofloxacin (data not shown); however, the SdeXY pump has been reported to efflux fluoroquinolones (Chen *et al.*, 2003), which could account for the minor change in the ciprofloxacin accumulation for the knockout mutant strains in the absence and presence of CCCP. Ciprofloxacin accumulation for the *sdeB* and *hasF* knockout mutant strains (SDEAB1, HASF100) was very similar (Fig. 3) and both had a comparable susceptibility profile (Table 2), suggesting that the *S. marcescens* HasF is the only outer-membrane component of efflux pumps in this organism.

In conclusion, using knockout mutagenesis, we have established the importance of the *S. marcescens* SdeAB multidrug efflux pump as the primary RND pump responsible for increasing *S. marcescens* resistance to a range of compounds. In addition, we have established the role of HasF in contributing to the intrinsic resistance of *S. marcescens* to a variety of substances and the role of SdeR as an activator of the SdeAB efflux pump.

ACKNOWLEDGEMENTS

We are grateful to T. de Kievit for her valuable suggestions, A. Kumar and J. Manuel for their generous assistance and A. Ducas and L. Larsen for their technical support. This work was supported by a Natural Sciences and Engineering Research Council of Canada operating grant (to E. A. W.). S.B. is the recipient of a University of Manitoba Graduate Student Fellowship.

REFERENCES

- Alekshun, M. N. & Levy, S. B. (1997). Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. *Antimicrob Agents Chemother* **41**, 2067–2075.
- Aucken, H. M. & Pitt, T. L. (1998). Antibiotic resistance and putative virulence factors of *Serratia marcescens* with respect to O and K serotypes. *J Med Microbiol* **47**, 1105–1113.
- Barbosa, T. M. & Levy, S. B. (2000). Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. *J Bacteriol* **182**, 3467–3474.
- Buckley, A. M., Webber, M. A., Cooles, S., Randall, L. P., La Ragione, R. M., Woodward, M. J. & Piddock, L. J. V. (2006). The AcrAB-TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. *Cell Microbiol* **8**, 847–856.
- Chen, J., Kuroda, T., Huda, M. N., Mizushima, T. & Tsuchiya, T. (2003). An RND-type multidrug efflux pump SdeXY from *Serratia marcescens*. *J Antimicrob Chemother* **52**, 176–179.
- Chollet, R., Chevalier, J., Bollet, C., Pages, J. M. & Davin-Regli, A. (2004). RamA is an alternate activator of the multidrug resistance cascade in *Enterobacter aerogenes*. *Antimicrob Agents Chemother* **48**, 2518–2523.
- CLSI (2006). *Performance Standards for Antimicrobial Susceptibility Testing*. Supplement M100–S16. Wayne, PA: Clinical and Laboratory Standards Institute.
- Finan, T. M., Kunkel, B., De Vos, G. F. & Signer, E. R. (1986). Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J Bacteriol* **167**, 66–72.
- Fujimaki, K., Fujii, T., Aoyama, H., Sato, K.-I., Inoue, Y., Inoue, M. & Mitsuhashi, S. (1989). Quinolone resistance in clinical isolates of *Serratia marcescens*. *Antimicrob Agents Chemother* **33**, 785–787.
- Hachler, H., Cohen, S. P. & Levy, S. B. (1991). *marA*, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in *Escherichia coli*. *J Bacteriol* **173**, 5532–5538.
- Kaniga, K., Delor, I. & Cornelis, G. R. (1991). A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**, 137–141.
- Kumar, A. (2004). *Characterization of RND efflux pumps of Serratia marcescens*. PhD thesis, Department of Microbiology, University of Manitoba.
- Kumar, A. & Worobec, E. A. (2005a). Cloning, sequencing and characterization of the SdeAB multidrug efflux pump of *Serratia marcescens*. *Antimicrob Agents Chemother* **49**, 1495–1501.
- Kumar, A. & Worobec, E. A. (2005b). HasF, a TolC-homolog of *Serratia marcescens*, is involved in energy-dependent efflux. *Can J Microbiol* **51**, 497–500.
- Lambert, H. P. & O'Grady, F. W. (1992). *Antibiotics and Chemotherapy*. Edinburgh: Churchill Livingstone.
- Li, X.-Z. & Nikaido, H. (2004). Efflux-mediated drug resistance in bacteria. *Drugs* **64**, 159–204.
- Ma, D., Cook, D. N., Alberti, M., Pon, N. G., Nikaido, H. & Hearst, J. E. (1995). Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. *Mol Microbiol* **16**, 45–55.
- Maxwell, A. & Lawson, D. M. (2003). The ATP-binding site of type II topoisomerases as a target for antibacterial drugs. *Curr Top Med Chem* **3**, 283–303.
- Mortimer, P. G. & Piddock, L. J. (1991). A comparison of methods used for measuring the accumulation of quinolones by *Enterobacteriaceae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *J Antimicrob Chemother* **28**, 639–653.
- Nagakubo, S., Nishino, K., Hirata, T. & Yamaguchi, A. (2002). The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. *J Bacteriol* **184**, 4161–4167.
- Pearson, J. P., Pesci, E. C. & Iglewski, B. H. (1997). Roles of *Pseudomonas aeruginosa* *las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**, 5756–5757.
- Posadas, D. M., Martin, F. A., Sabio y Garcia, J. V., Spera, J. M., Delpino, M. V., Baldi, P., Campos, E., Cravero, S. L. & Zorreguieta, A. (2007). The TolC homologue of *Brucella suis* is involved in resistance

to antimicrobial compounds and virulence. *Infect Immun* **75**, 379–389.

Pumbwe, L. & Piddock, J. V. (2002). Identification and molecular characterization of CmeB, a *Campylobacter jejuni* multidrug efflux pump. *FEMS Microbiol Lett* **206**, 185–189.

Ruzin, A., Keeney, D. & Bradford, P. A. (2007). AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline

in *Acinetobacter calcoaceticus*–*Acinetobacter baumannii* complex. *J Antimicrob Chemother* **59**, 1001–1004.

van Amsterdam, K., Bart, A. & van der Ende, A. (2005). A *Helicobacter pylori* TolC efflux pump confers resistance to metronidazole. *Antimicrob Agents Chemother* **49**, 1477–1482.

Edited by: P. Cornelis