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Molecular cloning and characterization of SmrA, a novel ABC multidrug efflux pump from *Stenotrophomonas maltophilia*

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***Objectives*: *Stenotrophomonas maltophilia* is an emerging nosocomial pathogen that can cause difficult-to-treat infections and exhibits significant degrees of poorly understood multidrug resistance (MDR). The aim of this study was to identify and characterize a multidrug ATP-binding cassette (ABC) efflux pump in *S. maltophilia*.**

***Methods*: SmrA was identified in the *S. maltophilia* genome based on the detection of ABC transporter conserved motifs and alignment with experimentally proven MDR ABC transporters. The *smrA* gene was cloned and expressed in the hypersusceptible *acrAB* mutant *Escherichia coli* strain SM1411. The resistance to several antimicrobial agents was tested using Stokes' disc diffusion and broth microdilution MIC methods. Norfloxacin accumulation and efflux assays were performed using a fluorescence method with and without the efflux pump inhibitors sodium *O*-vanadate and reserpine.**

***Results*: Cloning and expression of *smrA* in *Escherichia coli* conferred increased resistance to structurally unrelated compounds, including fluoroquinolones, tetracycline, doxorubicin and multiple dyes. Moreover, the expression of *smrA* in *E. coli* reduced norfloxacin uptake and enhanced its efflux, features that could be inhibited by the ABC efflux pump inhibitors.**

***Conclusions*: SmrA is a member of the ABC multidrug efflux pump family. The findings warrant further study of the role of this molecule in *S. maltophilia* isolates, to estimate the potential impact of this system in antimicrobial resistance.**

Keywords: ABC transporters, multidrug exporter, antibiotic resistance, multidrug resistant

Introduction

The management of infections due to *Stenotrophomonas maltophilia* is greatly hampered by its intrinsic and acquired resistance to a wide variety of antimicrobial agents. It has thus emerged as an important nosocomial pathogen, able to colonize respiratory tract epithelial cells and the surfaces of medical devices, leading to pneumonia and bacteraemia. In one study, the mortality attributed to *S. maltophilia* ventilator-associated pneumonia in critically ill trauma patients was 23%, similar to attributable mortalities of other Gram-negative bacteria.[1] In addition to enzyme-mediated resistance, overexpression of efflux pumps is thought to be an important factor in the multiple resistance phenotype of *S. maltophilia*.[2] For example, the overexpression of the resistance–nodulation–cell division efflux system, SmeDEF, has been associated with an increase in the MICs of several antibiotics.[3] While the majority of described efflux pumps in Gram-negative bacteria are driven by proton-motive force, multidrug ATP-binding cassette (ABC) transporters are rarely reported in such bacteria and their role in drug resistance might be underestimated.

Herein, we report the identification and characterization of a novel multidrug efflux pump of the ABC family in *S. maltophilia*.

Materials and methods

Identification of putative ABC transporter

Using bioinformatic approaches, based on detection of Walker A and Walker B motifs[4] and the ABC signature,[5] and targeting open reading frames (500–800 amino acids in length) that are composed

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of an N-terminal membrane-spanning domain and a C-terminal ABC domain, SmrA was identified in the *S. maltophilia* strain K279a genome.[2] Identity and similarity of SmrA to experimentally proven multidrug ABC transporters were estimated, namely the N-terminal and C-terminal halves of the human ABC transporter P-glycoprotein (MDR1),[6] LmrA of *Lactococcus lactis*[7] and VcaM of *Vibrio cholerae*.[8]

Molecular cloning and heterologous expression of SmrA

Oligonucleotide primers *smrA* forward 5′-ATGTTCCGTTGGTTTG AAT-3′ and reverse 5′-TTGATCGGCAGCAACAAA-3′ were designed to amplify the 1830 bp *smrA* putative efflux pump gene from *S. maltophilia* MAN05 (recovered from patient blood) using PCR (initial denaturation of 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1.5 min, and then an extension at 72°C for 7 min). The purified PCR product was cloned using the pBAD TOPO® TA Expression Kit (Invitrogen, Paisley, UK), following the manufacturer's protocol. The *lacZ* gene, from the control DNA template supplied with the kit, was cloned for use in control experiments. The *acrAB* mutant *E. coli* strain SM1411 (Δ*acrAB*::Tn903*kan*^{r})[9] was used as a host for gene cloning and subsequent expression of constructs. Expression from pBAD was induced by the addition of 1% L-arabinose (Sigma–Aldrich, Dorset, UK).

Antimicrobial susceptibility testing

Several antimicrobial agents were screened using Stokes' comparative disc diffusion method, with the test strain containing SmrA (*E. coli* SM1411/*smrA*) in the centre and the control strain containing the same plasmid cloned with the *lacZ* gene (*E. coli* SM1411/*lacZ*) on the outside. Mueller–Hinton agar (BD, Oxford, UK) supplemented with 1% L-arabinose (Sigma–Aldrich) was used, and all antimicrobial discs were supplied by Oxoid Ltd (Basingstoke, UK), including cefalexin, cefotaxime, cefoxitin, ceftazidime, cefepime, amoxicillin/clavulanic acid, piperacillin/tazobactam, ticarcillin/clavulanic acid, aztreonam, imipenem, meropenem, norfloxacin, ciprofloxacin, nalidixic acid, moxifloxacin, gentamicin, erythromycin, oleandomycin, chloramphenicol, minocycline, tetracycline, novobiocin, trimethoprim, sulfamethoxazole and polymyxin. All plates were incubated at 36°C in ambient air for 16–18 h. Antibiotics that showed a zone radius with *E. coli* SM1411/*smrA* smaller than the zone radius with *E. coli* SM1411/*lacZ* were selected for MIC testing. The MICs of doxorubicin, ofloxacin, antiseptics and several dyes were also determined. All MICs were determined by the broth microdilution method in cation-adjusted Mueller–Hinton broth containing 1% L-arabinose and following the CLSI recommended guidelines.[10] Antibiotics and dyes were purchased from Sigma–Aldrich.

Accumulation and efflux assays

Norfloxacin uptake and efflux assays in *E. coli* SM1411 harbouring *smrA* or *lacZ* were performed using fluorometric methods, essentially as described by Mortimer and Piddock.[11] Norfloxacin was added to a final concentration of 10 mg/L, and the fluorescence intensity was measured using a spectrofluorimeter (Jasco FP750, Great Dunmow, UK) at excitation and emission wavelengths of 281 and 446 nm, respectively. The efflux assays were performed by measuring the fluorescence of norfloxacin in the supernatant and pellet, and were repeated in the presence of different concentrations of efflux pump inhibitors. The supernatant assay was performed in the presence of different concentrations of sodium *O*-vanadate (Sigma–Aldrich), an ATPase inhibitor. During the equilibration step, sodium *O*-vanadate was added to final concentrations of 0.5, 1 and 2 mM, and the fluorescence intensity was measured in the supernatant. Likewise, reserpine (Sigma–Aldrich) was added to final concentrations of 5, 10 and 20 mg/L, but the fluorescence of the accumulated norfloxacin was measured from the lysed pellet.

Results and discussion

Sequence analysis of the smrA gene

Sequence analysis showed that SmrA is a polypeptide with 610 residues and a calculated molecular mass of 67.9 kDa, which has six predicted transmembrane segments in the N-terminal hydrophobic domain, followed by an ABC domain in the C-terminal hydrophilic region. The SmrA protein was found to be closely related to known multidrug ABC efflux proteins and shared significant similarities with the N-terminal (42.1%) and C-terminal (41.5%) halves of human P-glycoprotein (MDR1), LmrA (47.1%) and VcaM (70.8%). A BLAST search using the SmrA amino acid sequence against the National Center for Biotechnology Information (NCBI) database revealed significant similarity to proteins in various Gram-negative bacteria, including *Pseudomonas aeruginosa* (81%) and *Acinetobacter* spp. (68%).

Antimicrobial susceptibility assays

Overexpression of *smrA* in *E. coli* SM1411, which is hypersusceptible to a number of antimicrobial agents due to a deficiency in the major multidrug efflux pump AcrAB, resulted in elevated MICs of a number of structurally dissimilar antimicrobial agents, indicating that SmrA is a multidrug resistance (MDR) protein. Amongst the 25 antibiotic discs tested, ciprofloxacin, norfloxacin and tetracycline showed a significant decrease (>3 mm) in the radius zone of *E. coli* SM1411/*smrA* compared with *E. coli* SM1411/*lacZ* (data not shown). Results of MIC testing showed an 8-fold increase in the MICs of ciprofloxacin, norfloxacin and tetracycline (Table 1).

Norfloxacin uptake

The uptake of norfloxacin was higher in *E. coli* SM1411/*lacZ* compared with *E. coli* SM1411/*smrA* (data not shown). As a result, the intracellular concentration of norfloxacin was lower throughout the experiment in the strain harbouring the *smrA* gene. This reduced intracellular concentration of the antibiotic may have significant clinical implications in strains overexpressing SmrA and might be associated with suboptimal therapy.

Extrusion of norfloxacin by SmrA and activity of efflux pump inhibitors

The effect of SmrA was further evidenced by the amount of norfloxacin extruded from cells. Figure 1(a) clearly shows that a higher concentration of norfloxacin was measured from the supernatant from the *E. coli* SM1411/*smrA* suspension compared with that from the *E. coli* SM1411/*lacZ* suspension, which may be attributed to a more efficient removal of the antibiotic by the SmrA

Table 1. Effect of *smrA* expression in *E. coli* SM1411 on susceptibility to a range of antimicrobial agents and dyes

Drug group and drug	MIC (mg/L)		Relative resistance[a]
	SM1411/*lacZ*	SM1411/*smrA*	
Antibiotics			
ciprofloxacin	0.03	0.25	8
norfloxacin	0.125	1	8
ofloxacin	0.125	0.25	2
tetracycline	0.125	1	8
doxorubicin	2	4	2
Dyes			
Hoechst 33342	0.5	1	2
rhodamine 6G	4	8	2
ethidium bromide	4	8	2
DAPI	2	4	2
TPPCI	4	8	2
Antiseptics			
chlorhexidine	2	2	1
triclosan	0.031	0.031	1

DAPI, 4′,6-diamidino-2-phenylindole; TPPCI, tetraphenyl phosphonium chloride.
[a]Relative resistance is the ratio of the MIC for SM1411/*smrA* to the MIC for SM1411/*lacZ*.

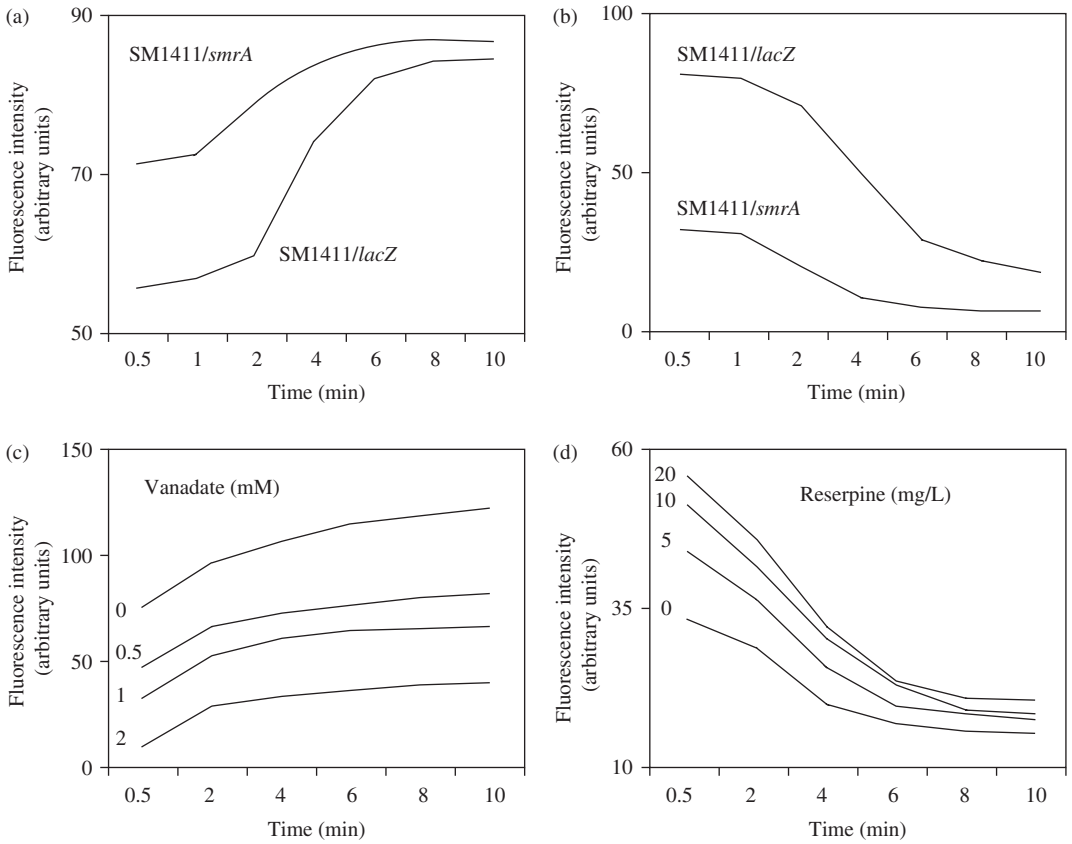

Figure 1. Efflux pump activity of SmrA as determined by the measurement of fluorescence intensity of norfloxacin in the supernatants (a) and pellets (b) derived from *E. coli* SM1411/*smrA* and *E. coli* SM1411/*lacZ* suspensions. The function of SmrA in *E. coli* SM1411/*smrA* was inhibited by sodium *O*-vanadate (c) and reserpine (d) in a concentration-dependent manner. The inhibition of efflux activities of SmrA was determined by measuring the fluorescence intensity of norfloxacin in the supernatant directly (c) and in the supernatant of the lysed pellet (d). Bacterial cells loaded with norfloxacin were prepared by incubating the suspensions with norfloxacin (10 mg/L) for 5 min, followed by washing and the addition of fresh buffer with or without different concentrations of the inhibitors.

protein. In addition, norfloxacin measured intracellularly was found to be consistently lower in *E. coli* SM1411/*smrA* compared with *E. coli* SM1411/*lacZ* (Figure 1b), supporting the suggestion that SmrA is a functionally active efflux pump. The efflux of norfloxacin was inhibited by the addition of sodium *O*-vanadate and reserpine in a concentration-dependent manner, confirming that SmrA is an ATP-dependent pump (Figure 1c and d). Neither sodium *O*-vanadate (2 mM) nor reserpine (20 mg/L) had an effect on the efflux of norfloxacin in the *E. coli* strain without *smrA*, i.e. *E. coli* SM1411/*lacZ* (data not shown).

Conclusions

We have shown that SmrA is a member of the ABC multidrug efflux pump family that is predicted to have six transmembrane helical regions followed by an ABC domain. Unlike P-glycoprotein, SmrA is a half-transporter that probably functions as a homodimer, and shares structural and functional similarity with each half of the human P-glycoprotein, and LmrA and VcaM. The expression of SmrA in a drug-hypersusceptible strain of *E. coli* conferred resistance to unrelated antimicrobial agents, indicating that the presence of SmrA in *S. maltophilia* may contribute to the intrinsic and/or acquired resistance of this important pathogen. Analysis of norfloxacin uptake and efflux provided direct evidence that SmrA is a membrane transporter that detoxifies the bacterial cells from these substrates. The inhibition of the efflux activity by sodium *O*-vanadate and reserpine provides evidence of the ATP-dependent nature of SmrA. The role of SmrA in its native host, *S. maltophilia*, is yet to be investigated.

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Transparency declarations

None to declare.

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