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Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides

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Objectives: Biocides and dyes are commonly employed in hospital and laboratory settings. We investigated the biocide susceptibilities of a rapidly emerging pathogen, *Acinetobacter baumannii*, and the underlying molecular mechanisms, with a primary focus on resistance–nodulation–cell division (RND) efflux systems.

Methods: Biocide susceptibilities, efflux and *in vitro* inactivation profiles were monitored in the presence/absence of efflux pump inhibitors. The RND transporters encoded by *adeB* and *adeJ* were detected by PCR; null mutants were constructed in the native host. Expression of *adeB* and *adeJ* in clinical isolates was assayed by semi-quantitative RT–PCR.

Results: Susceptibility testing and phenotypic assays demonstrated the role of active efflux in mediating decreased susceptibility to biocides. Inactivation of either the *adeB* or *adeJ* transporter gene led to increased susceptibility to biocides. RT–PCR analysis exhibited increased *adeB* and *adeJ* expression in clinical isolates.

Conclusions: This is the first study demonstrating the role of efflux pumps in mediating decreased susceptibility to disinfectants and other chemical substrates in *A. baumannii*.

Keywords: antimicrobial resistance, multidrug efflux, RND efflux pump

Introduction

Biocides are an integral component in the practice of clinical medicine, serving to prevent the dissemination of pathogenic organisms in the hospital environment.[1] Reduced susceptibility to biocides in bacterial species arises from various intrinsic and acquired (such as *qac* genes) genetic determinants, but efflux is increasingly implicated as a major resistance mechanism.[2] An association between resistance to antibiotics and cross-resistance to biocides has been reported for clinically important pathogens.[3]

One such important human pathogen is *Acinetobacter baumannii*, a Gram-negative bacillus that causes numerous healthcare-associated infections worldwide, with a remarkable propensity for nosocomial cross-transmission.[4] Reports have demonstrated the involvement of intrinsic and acquired resistance determinants as well as efflux pumps (AdeABC and AdeIJK) in conferring multidrug resistance.[5]

Despite the fact that these organisms have been linked to hospital environment contamination,[4,5] to date only a few studies have investigated the susceptibility profile of this bacillus to structurally unrelated compounds, antiseptics and biocides. The underlying genetic mechanisms responsible for mediating decreased susceptibility to disinfectants in *A. baumannii* still remain unknown. Prompted by the paucity of such information, a systematic study was initiated using 86 multidrug-resistant (MDR) isolates.

The biocide susceptibilities and underlying molecular mechanisms were investigated, with a primary focus on resistance–nodulation–cell division (RND) efflux systems. To our knowledge, this is the first study demonstrating the role of active extrusion and involvement of AdeABC and AdeIJK in mediating decreased susceptibility to biocides in *A. baumannii*.

Materials and methods

Bacterial strains, growth media and reagents

Eighty-six *A. baumannii* strains isolated during 2005–07 were obtained from two sources, i.e. the Ohio Department of Health ($n=38$) and The Ohio State University Medical Center ($n=48$).[6] All isolates were cultured in Luria–Bertani (LB) agar and LB broth (Difco, Sparks, MD, USA).

MICs

MICs were determined by a broth dilution method following CLSI guidelines.[7] The concentration with no visible growth was considered the MIC. The isolates were defined to exhibit decreased susceptibility if their MIC was found to be ≥2-fold higher than that for the *A. baumannii*

susceptible strain. The minimum bactericidal concentration (MBC) of biocides was determined by broth dilution in Mueller–Hinton broth (MHB; Difco), as described previously.[8] Biocides were diluted with broth for determination of MICs and this technique is known to have limitations. However, it is the method of choice for screening large numbers of isolates and was used in this study. The time–kill studies were performed according to CLSI guidelines.[7]

In vitro studies to elucidate the occurrence of active efflux

The accumulation of ethidium bromide (EtBr) or acriflavine (AF) and growth inhibition assays were examined as described previously, using the efflux pump inhibitors (EPIs) carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (an uncoupler of oxidative phosphorylation that disrupts the proton gradient of the membrane), phenylarginine-β-naphthylamide (PABN), reserpine and verapamil (Sigma, St Louis, MO, USA).[6] The antibacterial activity of EPIs has been investigated and it was found that EPIs have no intrinsic antibacterial activity against clinical isolates at the concentration (final concentration 25 mg/L) used in different experiments.

PCR amplifications and sequence analyses

Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). PCRs were performed using specific primers as described previously,[6,8] and confirmed by sequencing (CEQ 8000 capillary electrophoresis system; Beckman Coulter Instruments Inc., Palo Alto, CA, USA) and analysed by BLAST at www.ncbi.nlm.nih.gov. A detailed list of primers used for this study can be accessed at http://vet.osu.edu/IDMEL.

Construction of adeB and adeJ deletion mutants

The kanamycin resistance gene was retrieved from pUC4K upon digesting with BamHI and subsequently ligated with a pUC18 vector (New England Biolabs, MA, USA).

The resulting plasmid, pUC18Kan, was modified by cloning a PCR-amplified 0.981 or 1.8 kb internal fragment of *adeB* (5′-GTATG AATTGATGCTGC-3′ and 5′-CACTCGTAGCCAATACC-3′) or *adeJ* (5′-GCGAT AAAGTCATTGTTGATGGTG-3′ and 5′-CAGCATAGAGCACGCCAGAGAAG-3′), respectively, into the SmaI site of pUC18Kan. Obtained recombinant plasmids were introduced into clinical isolate AC0037 (kanamycin susceptible) by electrotransformation.[9,10] Ticarcillin- and kanamycin-resistant transformants were selected and the appropriate deletions were confirmed by PCR and genomic DNA sequencing. The resulting derivatives with inactivated *adeB* or *adeJ* were designated AC0037Δ*adeB*::*kan* and AC0037Δ*adeJ*::*kan*, respectively.

RT–PCR

To detect *adeB* and *adeJ* gene expression, RT–PCR was performed. Total RNA was extracted using an Absolutely RNA Miniprep Kit (Stratagene, La Jolla, CA, USA). Quantification of *adeB* and *adeJ* RNA transcripts was done using a Titan One Tube RT–PCR System (Roche Inc., USA). The primers used for *adeB* were 5′-GTATGAATTGATGCTGC-3′ and 5′-GACTTTCAGA TTCAAGATAT-3′, the primers used for *adeB* were 5′-GATATTGCACA GGTTCAAGTTCAAAACAAA-3′ and 5′-CCAAAGATATTAATGACCGCAAATGTA CCT-3′, and the primers used for 16s rRNA were 5′-TCGATGCAACGCGAA GAACC-3′ and 5′-CGTAAGGGCCATGATGACTT-3′. The bands were subjected to densitometric analysis using image scanning software Quantity One 4.1.1 (Bio-Rad). The expression levels were standardized relative to the transcription levels of 16s rRNA (a housekeeping gene) for each isolate. *P* values of <0.05 were considered statistically significant.

Results

Biocide susceptibility

Analysis of the MICs of different compounds clearly indicated that they varied. The clinical isolate *A. baumannii* AC0040 (reference for grouping) in our collection was an antibiotic- and biocide-susceptible strain.[6]

Therefore, for grouping, the MICs of benzalkonium chloride (BZK) and EtBr (two representative compounds) for 85 clinical isolates were compared with the respective MICs for *A. baumannii* AC0040 (Table 1). The isolates that exhibited 3-fold and 2-fold higher MICs of BZK and EtBr, respectively, with respect to that for the susceptible strain were classified as Group I. The isolates that exhibited 6-fold and >4-fold higher MICs of BZK and EtBr, respectively, were classified as Group II. The isolates that exhibited 24-fold and >8-fold higher MICs of BZK and EtBr, respectively, were classified as Group III (Table 1).

Of the total 86 isolates, 13% ($n=11$) of isolates had an MIC of BZK of <30 mg/L, 45% ($n=39$) of isolates had an MIC of 30 mg/L and 41% ($n=35$) had an MIC of 120 mg/L. About 67% of isolates had an MIC of AF of >200 mg/L (Table 1). The MICs of other compounds are shown in Table 1. Recently, we reported the resistance profiles of these clinical isolates and it was interesting to note that Group I isolates were either single drug resistant (chloramphenicol) or susceptible.[6] Isolates in Groups II and III were those that exhibited MDR, with MICs of ciprofloxacin being >32 or >72 mg/L, respectively.

The MBCs were determined for all the isolates. The MBC of a biocide is the concentration that kills 99.9% of the bacterial inoculum.[8] Data obtained in this study indicate that the MBCs of different biocides for Group III were higher than those for Groups I and II (Table 1). The susceptibility profiles of representative clinical isolates from Groups I, II and III to different biocides were found to be similar, both in terms of MBC testing and time–kill studies (data not shown).

Functional assay to demonstrate the occurrence of active efflux

Using the well-known efflux pump substrates EtBr and AF, a fluorimetric efflux experiment was performed on representative isolates from Groups II and III (high MICs) to elucidate the occurrence of active efflux. In the absence of CCCP, Group II/III strains accumulated 5- or 10-fold less EtBr, or AF, when compared with the susceptible strain. However, addition of CCCP increased the EtBr and AF accumulation in Group II/III strains, which eventually reached a plateau in all the strains (data not shown). These findings strongly demonstrate the occurrence of active efflux in these clinical isolates.

In vitro inactivation profiles

The effect of CCCP on the biocide susceptibility profile of the clinical isolates was tested. Interestingly, addition of CCCP (final concentration 25 mg/L) greatly reduced the MICs of various biocides from 2-fold up to 12-fold (Table 1). Reduction in the MICs of fluorescent dyes such as EtBr (17- to 26-fold) and AF (5- to 12-fold) was also observed (Table 1). Similar reductions in the MICs were found upon using PABN (data not shown). The EPIs

Table 1. Biocide susceptibility and carriage of efflux genes in clinical isolates of *A. baumannii*

			Biocides and dyes								Efflux genes		
Groups (*n*)[a]	*n*[a]		BZK[b]	CHX[b]	VKS[c]	Par[c]	Syn[c]	Wex[c]	EtBr[b]	AF[b]	*qacE*	*adeB*	*adeJ*
Susceptible	1	MIC	<5	<4	0.05	0.1	<0.005	0.01	<128	<12	−	−	+
		MBC	10	10	0.2	0.2	0.005	0.05	200	100			
I (11)	2	MIC[d]	15 (4)	16 (4)	0.1 (0.02)	0.4	0.005 (0.001)	<0.05	256 (10–30)	50 (10–20)	−	−	+
		MBC	20	20	0.4	0.8	0.005	0.05	400	200			
	7	MIC[d]	<30 (4)	16 (4)	0.15 (0.02)	0.4 (0.01)	0.005 (0.001)	<0.05	256 (10–30)	50 (10–20)	−	−	+
		MBC	20	20	0.4	0.8	0.005	0.05	400	200			
	2	MIC[d]	<30 (4)	16 (4)	0.15 (0.02)	0.4 (0.01)	0.005 (0.001)	<0.05	256 (10–30)	50 (10–20)	−	+	+
		MBC	20	20	0.4	0.8	0.005	0.05	400	200			
II (39)	16	MIC[d]	30 (6)	<24 (4)	0.2 (0.04)	0.6	0.05 (0.01)	0.05	512 (30–60)	120	−	−	+
		MBC	40	40	0.6	1.6	0.1	0.1	>800	>200			
	12	MIC[d]	30 (6)	32 (4)	0.2 (0.04)	0.8 (0.1)	0.1	0.08 (0.02)	800 (30–60)	>240 (20–40)	−	−	+
		MBC	40	60	0.6	3.2	0.2	0.2	>1600	>800			
	11	MIC[d]	30 (6)	32 (4)	0.2 (0.04)	0.8 (0.1)	0.1	0.08 (0.02)	800 (30–60)	>240 (20–40)	−	+	+
		MBC	40	60	0.6	3.2	0.2	0.2	>1600	>800			
III (35)	8	MIC[d]	120 (12–16)	64 (8–12)	<0.3 (0.08)	1 (0.1)	0.1 (0.01)	0.1 (0.02)	>1024 (60–120)	>240 (20–40)	+	+	+
		MBC	>160	80	0.8	3.2	0.2	0.2	>1600	>800			
	11	MIC[d]	120 (12–16)	>64 (10–12)	0.4 (0.08)	1 (0.1)	0.1 (0.01)	0.1 (0.02)	>1024 (60–120)	>240 (20–40)	+	+	+
		MBC	>160	80	1	3.2	0.2	0.2	>1600	>800			
	16	MIC[d]	120 (12–16)	100 (8–12)	0.4 (0.08)	1 (0.1)	0.1	0.2	>1024	>240	+	+	+
		MBC	>160	120	1	3.2	0.2	0.4	>1600	>800			

The different classes of disinfectant formulations used were: Virkon S (an oxidizing agent: peroxygen blend and organic acid; Antec International, UK); Wex-Cide 128 (a phenolic agent; Wexford Laboratories, USA); Synergize [a glutaraldehyde disinfectant-containing quaternary ammonium compound (QAC); Preserve International, USA]; chlorhexidine gluconate (MP Biomedicals, LLC); benzalkonium chloride (a QAC; MP Biomedicals, LLC); and Parvosol II RTU (a synergized QAC with inert ingredients; Hess & Clark Inc., USA).
BZK, benzalkonium chloride; CHX, chlorhexidine; VKS, Virkon S; Par, Parvosol; Syn, Synergize; Wex, Wex-Cide; EtBr, ethidium bromide; AF, acriflavine.
[a]*n*, Number of isolates.
[b]MIC and MBC values are represented as mg/L.
[c]MIC and MBC values are represented as %.
[d]Values in parentheses are MICs in the presence of the efflux pump inhibitor CCCP at 25 mg/L.

verapamil and reserpine had no effect on these isolates (data not shown). The EPIs (final concentration 25 mg/L) had no intrinsic antibacterial activity against the clinical isolates in this study.

In order to confirm the role of active efflux, we monitored the growth inactivation profiles of *A. baumannii* from Groups II and III in the presence and absence of EPIs using different concentrations of biocides. On the addition of CCCP, the susceptibilities of clinical isolates to biocides were highly increased (while no change in growth curve was found when verapamil and reserpine were used); we observed >5-fold decreases in MICs of chlorhexidine and Synergize, and 3-fold decreases in MICs of Virkon S and BZK (data not shown). These findings strongly imply that active efflux is involved in mediating high MICs of biocides.

PCR detection of efflux genes

PCR testing revealed that 41% (35/86) of the isolates harboured the chromosomal *qacE* disinfectant resistance gene (Table 1). None of the other *qac* alleles (*qacA*, *B*, *C*, *G*, *H* and *J*) were detected in this study. It is important to note that all *qacE*-positive isolates belonged to Group III.

Specific PCR assays were carried out to detect the efflux transporter encoded by *adeJ*, in an attempt to determine the distribution of this efflux system among different isolates with varying MICs of biocides. The *adeJ* gene was found in all Group I, II and III isolates (Table 1).

In our previous study, we found that *adeB*, *adeR* and *adeS* were present in 55% of the isolates in this collection.[6] The majority of the isolates that carried *adeB* belonged to Group III (Table 1). It is worthy to state here that the specific point mutations in *adeR* and *adeS* previously reported to cause AdeABC overexpression were not identified in these isolates.[6,9]

Role of adeABC and adeIJK in conferring antimicrobial resistance

To determine the contribution of *adeJ* and *adeB* efflux systems in disinfectant (biocide) susceptibility, *adeJ* or *adeB* was deleted in a clinical isolate, *A. baumannii* AC0037. Inactivation of either *adeB* or *adeJ* resulted in decreased susceptibility to different compounds. The following were the fold reductions in the MICs of various chemical agents in deletion mutants of *adeB* and *adeJ* genes, respectively: Acridine Orange (1, 4.4); acriflavine (8, 16); BZK (4, 6); chlorhexidine (8, 2); Crystal Violet (1, 10); 4′,6-diamidine-2-phenylindole (2, 4); deoxycholate (2, 2); EtBr (16, 8); Methyl Viologen (4, 16); pyronin Y (2, 4); rhodamine 123 (8, 16); SDS (6, 12); Synergize (2, 2); tetraphenylphosphonium chloride (12, 36); Virkon S (1, 1.5); and Wex-Cide (2, 2).

A fluorimetric experiment indicated that after 20 min (before adding CCCP) of incubation with EtBr, the Δ*adeB*::*kan* mutant showed a 2.2-fold increase in the rate of accumulation compared with its parental strain AC0037 (Figure 1a). Independent studies performed using the Δ*adeJ*::*kan* mutant demonstrated a 1.9-fold increase in the level of EtBr accumulation (Figure 1a). However, upon adding CCCP, the levels of accumulation in both parent and mutant strains were found to be almost similar (Figure 1a). In a separate set of experiments, the AF accumulation rates were also monitored. After 20 min, a 3-fold increase in AF accumulation was observed for the

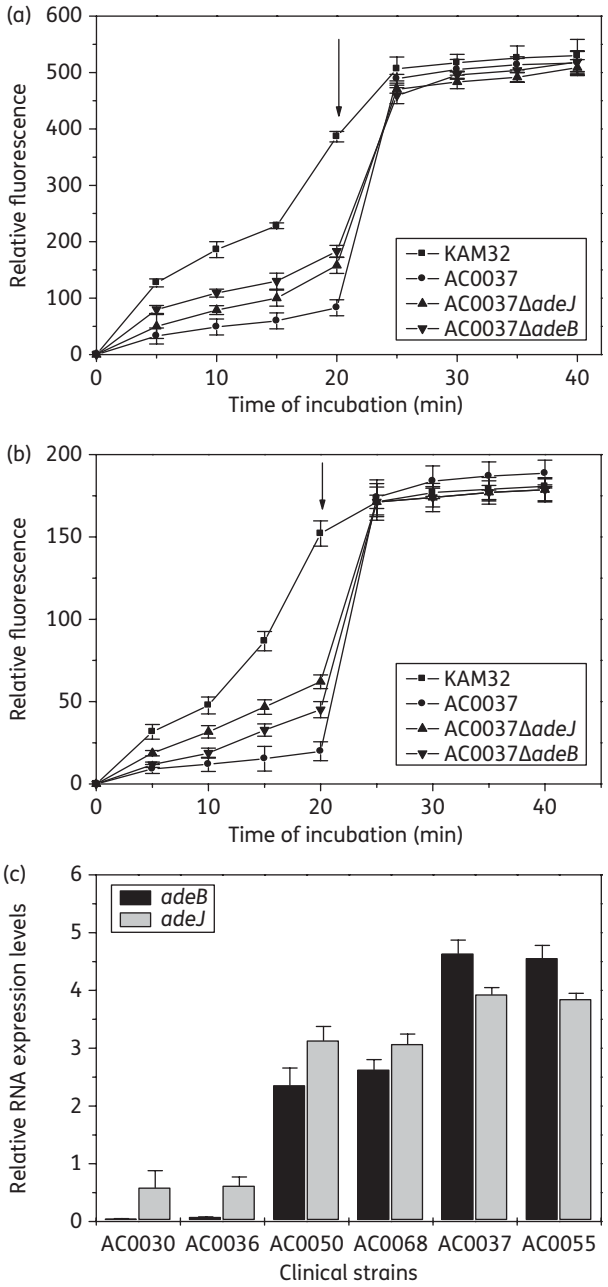

Figure 1. (a) Accumulation studies using ethidium bromide for wild-type *A. baumannii* and *adeB* and *adeJ* null mutants. The relative fluorescence intensity along the *y*-axis represents the level of accumulated ethidium bromide in the wild-type *A. baumannii*, isogenic mutants and *E. coli* KAM32. The graph shows the difference in the fluorescence shown by the bacterial cell in the presence and absence of the inhibitor CCCP. The arrow indicates the time of addition of CCCP to a final concentration of 25 mg/L. Each datapoint represents the mean ± SD of three independent experiments. (b) Accumulation studies using acriflavine. (c) Relative expression of *adeB* and *adeJ* genes from different clinical strains of *A. baumannii* (AC0030, AC0036, AC0050, AC0068, AC0037 and AC0055). The expression of the 16s rRNA gene was used as the internal control. Each bar represents the average value of two independent experiments and the error bars represent the standard deviations.

Δ*adeJ*::*kan* mutant compared with AC0037; however, a 2.2-fold increased accumulation rate was seen in the Δ*adeB*::*kan* mutant (Figure 1b).

Analysis of adeB and adeJ expression

We investigated the expression of membrane transporters encoded by *adeB* or *adeJ* in a range of isolates from all three groups using RT–PCR. Though the *adeB* gene was detected by PCR, we did not find any evidence for its expression in Group I (AC0030 and AC0036) strains. However, >4- and >8-fold increases in *adeB* expression were noticed in isolates from Groups II (AC0050 and AC0068) and III (AC0037 and AC0055), respectively (Figure 1c). The expression of *adeJ* was ∼6-fold higher in Group II and III isolates when compared with that of Group I isolates (Figure 1c).

Discussion

Considering the importance of disinfection in the prevention of nosocomial infection, the aim of this study was to evaluate the biocide susceptibilities of a set of MDR *A. baumannii* clinical isolates and to delineate the role of efflux pumps in mediating decreased biocide susceptibility. Notably, the majority of the isolates exhibited decreased susceptibility to various commonly used biocidal agents. Consistent with our findings, several studies have reported the increased resistance of nosocomial pathogens towards these agents.[4,5] It is important to state here that *A. baumannii* strains with decreased susceptibility to biocides may live through a non-optimal cleaning regimen or biocide challenge for a longer period, making them clinically more problematic to treat.

Phenotypic assays demonstrated that efflux pumps contribute as an important mechanism for the high-level biocide and antimicrobial resistance in *A. baumannii*. Various studies in Gram-negative bacteria have demonstrated that efflux pumps play an important role in intrinsic resistance to disinfectants including quaternary ammonium compounds.[3,4,8] The disinfectant gene *qacE* was found in more than one-third of the clinical isolates. Resistance to antiseptics and disinfectants associated with integrons carrying efflux-related transporters such as QacE has been well studied.[2]

Analysis indicated that the structurally unrelated compounds were also substrates for AdeIJK and AdeABC, and overexpression of these efflux pumps correlated well with the increased biocide MICs for Ohio clinical isolates. However, upon inactivating a single operon we did not observe a complete loss in resistance profile. Thus, it is important to state here that other efflux pumps in the genome of the bacteria may also have a role in conferring decreased susceptibility to biocides. With the concern that low-level biocide challenge can select for an MDR population, it is important to determine the susceptibility of clinical *A. baumannii* to various disinfectants and to promote strict intervention of control and preventive measures.

Conclusions

In this study, we have shown that *adeABC* and *adeIJK* transport systems can extrude disinfectants, structurally unrelated compounds and detergents, providing experimental evidence for their broad substrate specificity and an additional role in *A. baumannii*.

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Transparency declarations

None to declare.

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