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

Govindan Rajamohan1,2, Vijaya Bharathi Srinivasan1 and Wondwossen A. Gebreyes1*

1Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH, USA; 2Institute of Microbial Technology, Council of Scientific and Industrial Research, Sector 39A, Chandigarh, India

*Corresponding author. Tel: +1-614-292-9559; Fax: +1-614-292-4142; E-mail: gebreyes@cvm.osu.edu

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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.6 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), phenyl-arginine-β-naphthylamide (PABN), reserpine and verapamil (Sigma, St Louis, MO, USA). 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. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). PCRs were performed using specific primers as described previously. Genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA).

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’-GTATGATTAGTACGTCG-3’ and 5’-CAGCACGCAGACGCCGAGAAG-3’), respectively, into the SmaI site of pUC18Kan. Obtained recombinant plasmids were introduced into clinical isolate AC0040 (kanamycin susceptible) by electroporation.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 AC0037adeB::kan and AC0037adeJ::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’-GTATGATTAGTACGTCG-3’ and 5’-GACCTTTCGATCAGAT-3’, the primers used for adeJ were 5’-GATTGCAAGTAAACACCG-3’ and 5’-CCCAAATGAAATTGAGCGC-3’, respectively, into the SmaI site of pUC18Kan. Obtained recombinant plasmids were introduced into clinical isolate AC0040 (kanamycin susceptible) by electroporation.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 AC0037adeB::kan and AC0037adeJ::kan, respectively.

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 MIC of a biocide is the concentration that kills 99.9% of the bacterial inoculum.8 Data obtained in this study indicate that the MICs 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 beautiful 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>
<thead>
<tr>
<th>Groups (n)</th>
<th>MIC</th>
<th>MBC</th>
<th>BZK</th>
<th>CHX</th>
<th>VKS</th>
<th>Par</th>
<th>Syn</th>
<th>Wex</th>
<th>EtBr</th>
<th>Af</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>1</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.005</td>
<td>0.05</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>I (11)</td>
<td>2</td>
<td>20</td>
<td>20</td>
<td>0.4</td>
<td>0.8</td>
<td>0.005</td>
<td>0.05</td>
<td>400</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>II (39)</td>
<td>16</td>
<td>40</td>
<td>40</td>
<td>0.6</td>
<td>1.6</td>
<td>0.1</td>
<td>0.1</td>
<td>&gt;800</td>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>III (35)</td>
<td>8</td>
<td>&gt;160</td>
<td>120</td>
<td>64</td>
<td>8</td>
<td>&lt;0.3</td>
<td>1</td>
<td>0.1</td>
<td>&gt;1024</td>
<td>&gt;240</td>
</tr>
</tbody>
</table>

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, acriflavin.

<table>
<thead>
<tr>
<th>Efflux genes</th>
<th>qacE</th>
<th>adeB</th>
<th>adeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>I (11)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>II (39)</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>III (35)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

n, Number of isolates.

MIC and MBC values are represented as mg/L.

MIC and MBC values are represented as %.

Values in parentheses are MICs in the presence of the efflux pump inhibitor CCCP at 25 mg/L.
were present in 55% of the isolates in this collection. The distribution of this efflux system among different isolates with I, II and III isolates (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

**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. 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); and Wex-Cide (2, 2).

A fluorometric experiment indicated that after 20 min (before adding CCCP) of incubation with EtBr, the ΔadeB::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 ΔadeJ::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

![Graph](image-url)
\(\Delta adeJ::\text{kan} \) mutant compared with AC0037; however, a 2.2-fold increased accumulation rate was seen in the \(\Delta adeB::\text{kan} \) 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.\(^{2-5}\) 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 antisepsics 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.

**References**