Prevalence of eight resistance-nodulation-division efflux pump genes in epidemiologically characterized Acinetobacter baumannii of worldwide origin

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The increasing emergence of multidrug-resistant Acinetobacter baumannii constitutes a worldwide threat in hospital settings. Efflux-mediated resistance, particularly the resistance-nodulation-division (RND)-type efflux pumps, contributes significantly to decreased susceptibility to multiple antibiotics when overexpressed. Using PCR-based detection, the prevalence of genes encoding the RND efflux pumps AdeB, AdeJ and AdeG was investigated amongst 144 epidemiologically characterized and geographically diverse A. baumannii isolates of worldwide origin, representing International Clones 1–8 and genotypically unique isolates. Furthermore, five putative RND-type efflux genes identified via an in silico approach were included. Five of the eight investigated efflux pump genes were present in all isolates, including adeJ and adeG; the prevalence of the others varied between 65 and 97%. No association between the presence of one or multiple pumps to a specific clonal lineage was detected. The high prevalence of the efflux pump genes supports a fixed function of each individual pump that is not yet fully understood.

INTRODUCTION

Acinetobacter baumannii is a non-fermenting Gram-negative micro-organism, whose clinical importance has significantly increased worldwide over the past two decades (Peleg et al., 2008; Villegas & Hartstein, 2003). Establishing itself a niche in hospital settings, this opportunistic pathogen is involved in serious nosocomial infections, such as ventilator-associated pneumonia, meningitis, and bloodstream, urinary tract and wound infections, especially in patients with severe co-morbidities in the intensive care unit setting (Munoz-Price & Weinstein, 2008). Due to its intrinsic antimicrobial resistance and its ability to easily acquire new resistance determinants (Coyne et al., 2011; Dijkshoorn et al., 2007), the prevalence of A. baumannii isolates displaying resistance to multiple antibiotics has increased dramatically and only limited options remain for the treatment of these infections (Montero et al., 2004). In Gram-negative bacteria, various mechanisms are associated with a multidrug resistance (MDR) phenotype, including enzymic drug inactivation/modification, target alteration, low membrane permeability and active efflux. Of these, increased expression of chromosomally encoded efflux pumps has been proposed as the first step in the development of a MDR phenotype (Piddock, 2006) and several reports have been published showing decreased susceptibility to multiple antibiotics due to the overexpression of normally cryptic efflux systems in A. baumannii (Coyne et al., 2010; Damier-Piolle et al., 2008; Higgins et al., 2010a; Magnet et al., 2001).

Efflux pumps are components of the bacterial membrane that excrete metabolic end-products and toxic substances, including antimicrobials (Helling et al., 2002). Thereby, they show a broad substrate specificity, exporting multiple, structurally unrelated compounds out of the bacteria (Piddock, 2006). As the efflux system most often associated with MDR in A. baumannii when overexpressed, the resistance-nodulation-division (RND)-type family is of particular clinical importance. The RND efflux complex is composed of a cytoplasmic membrane spanning transporter protein interacting with an outer membrane pore [outer membrane protein (OMP)] and a periplasmic membrane fusion protein (MFP) to facilitate the drug transport across the outer membrane (Tseng et al., 1999; Zgurskaya & Nikaido, 2000). The proton gradient across the membrane serves as a driving force. To date, three RND efflux
systems have been described in *A. baumannii*: AdeABC (Magnet *et al.*, 2001), AdeFGH (*Coyne et al.*, 2010) and AdeJK (*Damier-Piolle et al.*, 2008).

Only few data about the prevalence of RND efflux pumps in *A. baumannii* exist. Nemec *et al.* (2007) reported that the *adeB* gene was present in 87% of 116 genetically diverse predominantly European *A. baumannii* isolates. Other studies dealt with genetically related isolates (Huys *et al.*, 2005b) or worked with a collection from one particular hospital (Chu *et al.*, 2006; Lin *et al.*, 2009).

In this study, we investigated genotypically and geographically diverse *A. baumannii* isolates collected worldwide (Halstead *et al.*, 2007), representing International Clones IC1–8 and genotypically unique isolates (Higgins *et al.*, 2010b) to detect the RND efflux pump genes *adeB*, *adeG* and *adeJ*. In addition, using the published *A. baumannii* genomes, we identified another five RND efflux pumps, which were included in the study. The objective of this study was to investigate the prevalence of these eight RND pump genes in epidemiologically characterized clinical *A. baumannii* isolates.

**METHODS**

**Bacterial isolates.** In total, 144 multidrug-resistant *A. baumannii* isolates were chosen to cover a wide geographical origin and epidemiological background. The isolates were recovered from 68 centres in 26 countries in Europe, North and South America, Asia, and Africa (Higgins *et al.*, 2010b), and previously typed using DiversiLab. The collection included isolates belonging to IC1–8 (N=11, 38, 9, 16, 16, 11, 5 and 10 isolates, respectively) and genotypically unique isolates (N=28). Multiple isolates from single centres were only included where multiple clonal lineages were present. All isolates, with the exception of three, were carbapenem resistant and therefore exhibited a high clinical relevance.

**Identification of putative RND efflux pump genes.** An in silico approach was used to identify putative RND pumps in *A. baumannii*. ORFs from published genomes in the National Center for Biotechnology Information (NCBI) database (*A. baumannii* ACICU, AB0057 and ATCC17978) were analysed for RND-type efflux pumps by a BLAST search using *A. baumannii* pump *AdeB* (GI:1618478) as the search query. To determine the prevalence of the putative RND pumps amongst all published complete *A. baumannii* genomes, a BLAST search using the amino acid and nucleotide sequence of the putative pumps was performed.

**Detection of RND efflux pump genes.** All primers used in this study were designed using Primer3 software unless otherwise stated (http://bioinfo.ut.ee/primer3/). The presence of genes encoding the efflux pumps *AdeB*, *AdeG* and *AdeJ* and five putative RND pumps identified in silico was investigated by PCR. Primers are listed in Table 1. To avoid false-negative results due to small genetic variations of DNA regions targeted by the first primer pair, a second primer pair was created for each gene where necessary.

PCR reactions contained 12.5 of µl of *Taq* PCR Master Mix (Qiagen), 5 pmol of each primer and 1 µl of heat-lysed bacteria were performed in a final volume of 25 µl. The following parameters were used for thermal cycling: initial denaturation at 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 55 °C for 20 s and 72 °C for 1 min, and final elongation at 72 °C for 5 min. PCR products were analysed on agarose gels, stained with ethidium bromide and visualized on a UV transilluminator.

**RESULTS**

BLAST analysis identified five putative RND pumps in the three *A. baumannii* genomes with the *A. baumannii* ACICU accession numbers ACICU_02904, ACICU_00143, ACICU_03412, ACICU_03066 and ACICU_03646 (Table 2). ACICU_02904, annotated as a cation/multidrug efflux pump, was 1021 aa in size and was 40% identical to the *AdeB* amino acid sequence. No further genes characteristic for the RND-type complex (MFP, OMP) were encoded immediately upstream or downstream of the pump. The same was true for the RND superfamily exporter ACICU_00143, 1216 aa in size and 23% identical to the *AdeB* amino acid sequence. For both RND family cation/multidrug efflux pumps ACICU_03066 and ACICU_03646, a MFP was encoded upstream of the pump. Additionally, in the case of ACICU_03646, a TetR-type transcriptional regulator was found adjacent to the MFP. The latter pump exhibited 1041 aa and was 24% identical to the *AdeB* amino acid sequence, whereas ACICU_03066 was 1011 aa in size and displayed 29% similarity to *AdeB*. Regarding the efflux transporter ACICU_03412, both the MFP and OMP were encoded upstream of the pump displaying a tripartite RND efflux system. Similar to *adeAB*, the start and the stop codon of the genes encoding the MFP and the pump ACICU_03412 overlapped, which is indicative of an operon organization.

The prevalence of the eight assigned RND efflux pumps amongst *A. baumannii* genomes in silico was detected using the NCBI database. Twenty-five complete *A. baumannii* genomes were available to compare against *A. baumannii* ACICU. Unassembled whole-genome-shotgun sequences were not considered as complete genomes as they might have gaps and thus sequence validity was not given. Due to their incomplete sequence, draft genomes were also excluded. Efflux pumps showing >95% sequence similarity were rated as present in the query isolate. Under these conditions, *adeG*, *adeJ*, ACICU_03066 and ACICU_03646 were present in all of the 25 fully sequenced *A. baumannii* genomes. Twenty-four isolates (96%) harboured ACICU_2904 and ACICU_00143, whereas 23 (92%) harboured *adeB*. In contrast, ACICU_03412 was present in less than half of the isolates (44%). The results were the same regardless of whether the amino acid sequence or the nucleotide sequence was compared.

The PCR-based detection of the eight RND efflux pump genes amongst our *A. baumannii* collection revealed that five of the pumps (*adeG*, *adeJ*, ACICU_02904, ACICU_03066 and ACICU_03646) were present in all of the *A. baumannii* isolates (Table 3). *AdeB*, the pump most often associated with MDR, was present in 97% of all isolates; one isolate each in IC5 and IC8, and three of the genotypically unique isolates were missing the gene. Similarly, the uncharacterized ACICU_00143 was present...
Table 1. Primers used for the detection of the efflux pump genes

<table>
<thead>
<tr>
<th>Efflux pump gene</th>
<th>Primer name</th>
<th>Primer sequence (5’→3’)</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>adeB</td>
<td>O3*</td>
<td>GTATGAAATTGATGCTGC</td>
<td>981</td>
</tr>
<tr>
<td></td>
<td>O4*</td>
<td>CACTCGTACCCAATACC</td>
<td></td>
</tr>
<tr>
<td>adeB</td>
<td>adeB 2_F</td>
<td>GAATAAGGCAACGCAAAAT</td>
<td>124</td>
</tr>
<tr>
<td>adeB</td>
<td>adeB 2_R</td>
<td>TTGCGAATCAGTGTTTCCA</td>
<td></td>
</tr>
<tr>
<td>adeG</td>
<td>adeG 1_F</td>
<td>TGAACGATGCTGCTAAAC</td>
<td>681</td>
</tr>
<tr>
<td>adeG</td>
<td>adeG 1_R</td>
<td>CTCCAGCTGTCAACGAGCA</td>
<td></td>
</tr>
<tr>
<td>adeG</td>
<td>adeG 2_F</td>
<td>GGATTTGATGGCGATTAGC</td>
<td>134</td>
</tr>
<tr>
<td>adeG</td>
<td>adeG 2_R</td>
<td>TGATGGGCTTTCCAAATTCAC</td>
<td></td>
</tr>
<tr>
<td>adeJ</td>
<td>adeJ 1_F</td>
<td>CTGGTGATGATCAGGGATT</td>
<td>605</td>
</tr>
<tr>
<td>adeJ</td>
<td>adeJ 1_R</td>
<td>TGAACCAAGACTCAGGTC</td>
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</tr>
<tr>
<td>ACICU_02904†</td>
<td>ACICU_02904 1_F</td>
<td>ATGACGGCATTTGTTGTA</td>
<td>623</td>
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<td></td>
<td>ACICU_02904 1_R</td>
<td>CTTGCGCTAAATATTCAC</td>
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<td></td>
<td>ACICU_02904 2_R</td>
<td>ATATGGGCGACTGTGCTCA</td>
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</tr>
<tr>
<td>ACICU_00143†</td>
<td>ACICU_00143 1_F</td>
<td>TTCCGCTCAATATTCGCA</td>
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<tr>
<td></td>
<td>ACICU_00143 1_R</td>
<td>AGTGTGGTGTTGCTGACG</td>
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<td>ACICU_00143 2_F</td>
<td>GTAAGGATGGCGGATTTGC</td>
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<td></td>
<td>ACICU_00143 2_R</td>
<td>TGCAGCGAATGAGAGAGAG</td>
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<tr>
<td>ACICU_03412†</td>
<td>ACICU_03412 1_F</td>
<td>TATGGGCTTTCCAAATTCAC</td>
<td>734</td>
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<tr>
<td></td>
<td>ACICU_03412 1_R</td>
<td>CGGTTCATACACCGTCTGAT</td>
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<td>ACICU_03412 2_F</td>
<td>ACAAATGGGTGTGAAAGCA</td>
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<td></td>
<td>ACICU_03412 2_R</td>
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<tr>
<td>ACICU_03066†</td>
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<td>TCCGGCATGAAATGTGACA</td>
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<tr>
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<td>ACICU_03066 2_F</td>
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<tr>
<td></td>
<td>ACICU_03066 2_R</td>
<td>TTATAGGCCTGAGCGGAGA</td>
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<tr>
<td>ACICU_03646†</td>
<td>ACICU_03646 1_F</td>
<td>AGAATATGCGACTGTGTTG</td>
<td>793</td>
</tr>
<tr>
<td></td>
<td>ACICU_03646 1_R</td>
<td>AATTGCGCTTATACCCCTTG</td>
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</tbody>
</table>

*Previously described by Magnet et al. (2001).
†Locus tag in A. baumannii ACICU.

Table 2. Features of the five uncharacterized RND efflux pump genes

<table>
<thead>
<tr>
<th>Locus tag</th>
<th>Annotated as</th>
<th>Size (aa)</th>
<th>Features</th>
<th>Amino acid identity to AdeB (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACICU_02904</td>
<td>Cation/multidrug efflux pump</td>
<td>1031</td>
<td>Upstream: glycerophosphoryl diester phosphodiesterase,</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>zinc-dependent hydrolase Downstream: HptX, hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>ACICU_00143</td>
<td>RND superfamily exporter</td>
<td>1216</td>
<td>Upstream: polyketide synthase module, non-ribosomal peptide synthesis protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Downstream: hypothetical protein, NAD-dependent epimerase</td>
<td>23</td>
</tr>
<tr>
<td>ACICU_03412</td>
<td>Putative silver efflux pump</td>
<td>1052</td>
<td>Upstream: MFP, OMP Downstream: hypothetical protein, cobalt/zinc/cadmium</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>efflux system component</td>
<td></td>
</tr>
<tr>
<td>ACICU_03066</td>
<td>Cation/multidrug efflux pump</td>
<td>1011</td>
<td>Upstream: hypothetical protein (nodulation protein), MFP Downstream:</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>prolyl-tRNA synthetase, hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>ACICU_03646</td>
<td>Cation/multidrug efflux pump</td>
<td>1041</td>
<td>Upstream: MFP, transcriptional regulator (TetR) Downstream: chaperone protein DnaJ, hypothetical protein</td>
<td>24</td>
</tr>
</tbody>
</table>
in all *A. baumannii* isolates of IC2, IC3, IC6 and IC7, whereas one to three isolates were negative for this pump in IC1, IC4, IC5 and the genotypically unique isolates. In IC8, <50% of the isolates were positive for this gene. The highest diversity in the prevalence of the eight investigated RND efflux pumps was observed with ACICU_03412; ≥90% of the IC1, IC3, IC6 and IC8 isolates carried the gene, whilst ~60% of the genotypically unique isolates and IC5 gave a positive PCR product. Furthermore, 40% of isolates in IC7, 29% in IC2 and 6% in IC4 amplified ACICU_03412. Overall, there was no association between the presence of one or multiple pumps to a specific clonal lineage. However, all isolates of IC3 and IC6 were positive for all the genes investigated. With an overall prevalence of 56%, ACICU_03412 was detected least of all (for IC2, IC4 and IC7 the prevalence was <50%).

The total prevalence of the efflux pumps amongst our strain collection showed a high similarity to the prevalence in the publicly available completely sequenced *A. baumannii* genomes in the NCBI database. Therefore, our PCR-based method to determine the prevalence of RND-type efflux pumps was reliable. In addition, BLAST analysis of whole genomes revealed that polymorphisms within the primer-binding sites were rare.

**DISCUSSION**

We report that RND-type efflux systems are widely distributed in *A. baumannii*: five of eight RND efflux genes were present in all 144 tested isolates, and the other three genes had a prevalence varying between 56 and 97%.

To our knowledge, this is the first report describing the distribution of both characterized and uncharacterized RND efflux pumps in a genotypically diverse pool of clinically relevant *A. baumannii* isolates. Previously, Nemec *et al.* (2007) found that 82% of *A. baumannii* isolates from a predominantly European collection carried *adeB*. In another European study, 49 out of 51 isolates divided into European Clones 1 and 2 (which correspond to IC1 and IC2, respectively) and genotypically unique isolates harboured *adeB* (Huys *et al.*, 2005b). Chu *et al.* (2006) reported the presence of three efflux systems in different *Acinetobacter* genomic DNA groups. In their collection of 59 *A. baumannii* isolates, 70% were positive for *adeB* by PCR (Chu *et al.*, 2006). However, these isolates came from only one hospital in Hong Kong. Under similar conditions, Lin *et al.* (2009) reported that 84 and 90% of their 112 isolates carried *adeB* and *adeJ*, respectively.

In these previous studies, only one primer pair was used. However, if there are nucleotide polymorphisms within the primer annealing site that lead to no amplicon, a false-negative result can occur. The finding of 11 *adeB* sequence types amongst 50 *A. baumannii* isolates belonging to European Clones 1–3 (IC1–3) demonstrates the sequence variability in this gene (Huys *et al.*, 2005a). For this reason, we used two separate primer pairs per gene, where necessary. For *adeJ* and ACICU_03646, no second primer pair was needed; a positive amplicon was detected for all isolates with the first primer set used. In the case of *adeB* and *adeG*, five isolates had to be retested with the second primer pair, which revealed that all isolates were positive for *adeG*, whereas the *adeB* gene was missing in four isolates. However, the use of both primer sets was frequently needed for the remaining efflux pump genes. In 66% of cases in which a negative result was obtained with the first pair of primers, using the second pair gave a positive result. Nevertheless, whilst it is possible that nucleotide polymorphisms within the second primer annealing sites could result in a false-negative result, when we analysed available genome sequences, these polymorphisms were rare.

We found no association between the epidemiological background of an isolate and its efflux gene profile. It is noteworthy that five of the eight investigated RND family genes were present in all isolates tested. The other three pumps, including *adeB*, showed a high prevalence in our diverse pool of isolates. As bacterial genomes are highly

<table>
<thead>
<tr>
<th>Clone (no. of isolates)</th>
<th>adeB</th>
<th>adeG</th>
<th>adeJ</th>
<th>ACICU_02904*</th>
<th>Prevalence (%)</th>
<th>ACICU_00143*</th>
<th>ACICU_03412*</th>
<th>ACICU_03066*</th>
<th>ACICU_03646*</th>
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<tbody>
<tr>
<td>IC1 (11)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>82</td>
<td>91</td>
<td>100</td>
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<td>IC2 (38)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<td>Genotypically unique (28)</td>
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<td>89</td>
<td>61</td>
<td>100</td>
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<td>Total (144)</td>
<td>97</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>90</td>
<td>56</td>
<td>100</td>
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</table>

* Asterisk indicates a Locus tag in *A. baumannii* ACICU.
flexible and genes of no need or function are rapidly removed or replaced (Lan & Reeves, 2000), this finding supports a defined function of each individual pump that has yet to be determined. In Pseudomonas aeruginosa it has been shown that MexAB, MexCD, MexEF and MexXY each have fixed functions, i.e. MexCD functions as part of the envelope stress response (Fraud et al., 2008). However, MexEF is linked to the organism’s nitrosative stress response (Fetar et al., 2011), whereas oxidative stress (Fraud & Poole, 2011) activates MexXY expression. Except for the overlapping substrate profile of AdeB, Ade and AdeG, and a growth-phase-dependent expression of these genes (Fernando & Kumar, 2012), little is known about regulation of the RND-type efflux systems in A. baumannii. A synergy of efflux combined with other resistance mechanisms has been observed to achieve high-level antimicrobial resistance (Bratu et al., 2008; Higgins et al., 2010a; Luo et al., 2011). Further insight into the functionality of each pump, including a characterization of the reported putative RND-type efflux pumps, might reveal their individual importance in this organism.

In conclusion, the presence of the eight RND efflux pumps in our geographically and genotypically diverse collection of worldwide A. baumannii isolates varied between 56 and 100%. No association between IC clusters and the presence or absence of the pumps was observed. However, the fact that at least another five uncharacterized RND pumps are encoded on the chromosome illustrates the need for further investigations to gain a better understanding of their contribution to the ever-increasing emergence of multidrug-resistant A. baumannii.

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