Presence of active efflux systems AdeABC, AdeDE and AdeXYZ in different *Acinetobacter* genomic DNA groups

Two resistance–nodulation–cell division (RND) type active efflux systems, AdeABC and AdeDE, have been described in *Acinetobacter* (Chau *et al.*, 2004; Magnet *et al.*, 2001). Both systems confer upon the host a reduced susceptibility to aminoglycosides, fluoroquinolones, erythromycin, tetracycline and chloramphenicol. AdeABC differs from AdeDE by protecting the host from cefotaxime, whilst AdeDE increases the host resistance to ceftazidime and rifampicin (Chau *et al.*, 2004; Magnet *et al.*, 2001). The three genes encoding the tripartite AdeABC were found as an array but AdeDE has only the membrane fusion protein (MFP) gene (*adeD*) and the RND transporter gene (*adeE*) clustered together. The outer-membrane protein (OMP) for AdeDE has not been identified.

The clinical significance of differences in antimicrobial-susceptibility pattern among different *Acinetobacter* genomic DNA groups (GDGs) has been demonstrated (Houang *et al.*, 2003), and the importance of efflux systems in multidrug resistance in *Acinetobacter* is increasingly appreciated. Therefore, we applied specific PCR to detect the transporter genes *adeB* and *adeE* in our collection of *Acinetobacter* strains in an attempt to study the distribution of these two efflux systems among different GDGs.

Unexpectedly, a PCR designed to screen for *adeE* detected a variant in an *Acinetobacter* isolate belonging to GDG 3. Genome walking utilizing the commercial kit GenomeWalker (BD Biosciences Clontech) was employed to clone the flanking regions of the amplicon as described previously (Chau *et al.*, 2004). Alignment of 15 contigs generated after 12 rounds of walking yielded 3 consecutive ORFs of 1248, 3177 and 1452 bp (GenBank accession no. DQ223769). These three ORFs, theoretically encoding three peptides resembling an MFP (415 residues), an RND transporter (1058 residues) and an OMP (483 residues), were putatively designated

adeX, *adeY* and *adeZ*, respectively. When they were compared with other known RND efflux systems, AdeX had highest identity (46 %) to MexA in *Pseudomonas aeruginosa* (GenBank accession no. L11616), AdeY (60 %) to SmeE in *Stenotrophomonas maltophilia* (GenBank accession no. AJ746241) and AdeZ (41 %) to OprM in *P. aeruginosa* (GenBank accession no. AB011381), as determined by the software ClustalW (EBI). Conversely, AdeX had only 35 and 38 % peptide sequence identity to AdeA and AdeD, respectively; whereas AdeY had 45 and 51 % identity to AdeB and AdeE, respectively, and AdeZ had a relatively high identity, 67 %, to AdeC. Moreover, when the DNA sequence of the entire *adeXYZ* gene cluster was used to search against the genome of the strain ADP1, formally identified as *Acinetobacter calcoaceticus* GDG 1 (Barke *et al.*, 2004), a homologous gene cluster (2 872 149 to 2 878 057 bp) was found with an identity of 77 %; the identity of the theoretical peptides to AdeX, AdeY and AdeZ were 80, 89 and 87, respectively.

Twelve attempts were made to disrupt *adeY* by targeted integration, as previously described (Chau *et al.*, 2004), in order to assess the involvement of *adeY* in antimicrobial resistance. However, no stable transformant was obtained and the reason behind the repeated failures was not known. Although the role of AdeXYZ in antimicrobial resistance has not been proven, given its increased similarity in loci organization and peptide sequences to known RND drug efflux systems, the possibility of its involvement cannot be ruled out. Furthermore, hydrophobicity analysis of the theoretical peptide sequence using the software TopPred (Pasteur Institut) demonstrated that AdeY has a topology highly compatible with it being an RND transporter (data not shown) (Tseng *et al.*, 1999).

Based on the new sequence information, new primer pairs AdeE-245A (5'-GTA GTA

GTT CGG CAG GAC AA-3') and AdeE-620B (5'-GCG GTT CTA ACA TCT GAT GG-3'), and AdeY-343A (5'-CAA TCT GCA ACT GCG CTT-3') and AdeY-920B (5'-TCA ACA GCT TCT GCG GTA-3'), were designed to detect the presence of *adeE* (expected amplicon size 376 bp) and *adeY* (expected amplicon size 587 bp), respectively. Primers O3 and O4, described by Magnet *et al.* (2001), were used to screen for *adeB*. A total of 207 *Acinetobacter* blood culture isolates obtained between 1997 and 2000 in our hospital were screened for the presence of *adeB*, *adeE* and *adeY*. The GDGs of all the isolates were determined by amplified rDNA restriction analysis as described elsewhere (Vanechoutte *et al.*, 1995).

Of the 59 GDG 2 (*Acinetobacter baumannii*) isolates tested, 39 (70 %) were positive with *adeB* PCR. The *adeB* sequence was not detected in any of the other GDGs, suggesting that *adeB* is an active efflux system specific to GDG 2 (Table 1). For the *adeE* and *adeY* PCR, 70 (70 %) and 75 (90 %) out of 83 GDG 3 isolates were positive, respectively, whilst all of the GDG 2 isolates tested were negative. Furthermore, *adeE* and *adeY* were found not only closely associated with GDG 3, but also coexisting in many isolates, including one GDG 13TU isolate and one GDG 17 isolate (Table 1). Of the 83 GDG 3 isolates tested, 68 (82 %) possessed both *adeE* and *adeY* sequences, whilst just 2 (2 %) had *adeE* only and 7 (8 %) had *adeY* only. The identity of all the amplicons was confirmed by RFLP analysis with restriction enzymes, *HhaI*, *HinfI*, *HincII* and *MseI* for *adeX*, and *HhaI*, *HinfI*, *HincII*, *ApaI* and *RsaI* for *adeE* (data not shown). The identity of 10 % of all the amplicons was reconfirmed by DNA sequencing.

Our data demonstrates that *adeB* is present exclusively in GDG 2, whereas *adeE* and *adeY* are present most frequently in GDG 3. Assuming the structure of all the three

Table 1. Presence of *adeB*, *adeE* and *adeY* in different *Acinetobacter* GDGs

GDG	Number of isolates			
	Total	<i>adeB</i>	<i>adeE</i>	<i>adeY</i>
2	56	39 (70 %)	0	0
3	83	0	70 (84 %)	75 (90 %)
5	7	0	0	0
10	4	0	0	0
12	2	0	0	0
13TU	24	0	1* (4 %)	1* (4 %)
13BJ	20	0	0	0
15	5	0	0	0
16	1	0	0	0
17	5	0	1† (20 %)	1† (20 %)

*These represent the same GDG 13TU isolate.
†These represent the same GDG 17 isolate.

gene clusters were preserved in the isolates, these results suggest that AdeABC is an active efflux system specific for GDG 2, whilst AdeDE and AdeXYZ are systems predominantly for GDG 3. The presence of both *adeE* and *adeY* in one GDG 13TU isolate and one GDG 17 isolate suggested similar if not identical systems can exist in other GDGs, and the presence of a gene cluster homologous to *adeXYZ* in ADP1 (formerly classed as GDG 1, but currently unclassified) also lends support to this notion. Further characterization of the region flanking these two genes in these two isolates could yield more information on the relatedness of these systems in different GDGs. The significance of the increased frequency of co-existence of AdeDE and AdeXYZ is not known. Unfortunately, the importance of AdeXYZ in antimicrobial resistance has not been ascertained since the targeted disruption of *adeY* has not been successful. This repeated failure in

obtaining an *adeY*-disrupted transformant suggested *adeY* could have an essential role in cell functions other than antimicrobial resistance. Nevertheless, given GDG 3 is one of the most clinically relevant *Acinetobacter* GDGs worldwide, and especially in Hong Kong, as shown previously (Chu *et al.*, 1999), further investigation into this active efflux system is undoubtedly warranted.

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