significance of these enzymes lies in their function and not on their structure. Thus, reliance only on the sequences can sometimes be quite misleading, as shown by several examples of erroneous functional annotations of genes in genomic analysis.[14] In the first superfamily mentioned above, the sequence of the class D OXA-2 β-lactamase is more similar to that of the BlaR C-terminal domain than to that of the OXA-1 β-lactamase. On this basis, BlaR would be classified as a class D β-lactamase although its k_{cat} values for penicillins and cephalosporins are in the 10^{-6}–$10^{-5}\,s^{-1}$ range,[15] several orders of magnitude lower than the k_{cat} values exceeding $10^{2}\,s^{-1}$ reported for typical class D β-lactamases.[16] Similarly, in the second superfamily, the sequence of human glyoxalase II is closer to those of B1 and B2 enzymes than those of B3.[1] But glyoxalase should, nevertheless, not be classified as a metallo-β-lactamase.

In conclusion, there has been good acceptance of the structural classification schemes that recognize three families (or classes) of active-site serine enzymes and three (sub)-families of metalloenzymes. It seems unnecessary to make additional modifications to a well-established scheme without consideration of factors such as enzymatic mechanisms and functional behaviour.

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Journal of Antimicrobial Chemotherapy
doi:10.1093/jac/dki138
Advance Access publication 22 April 2005

New *aac*(6′)-*I* genes in *Enterococcus hirae* and *Enterococcus durans*: effect on β-lactam/aminoglycoside synergy

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Keywords: *E. hirae*, *E. durans*, aminoglycoside acetyltransferase enzymes, acetyltransferases, AAC(6′)-I, aminoglycoside/penicillin synergy

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Sir,

Enterococcus faecium species harbour an intrinsic *aac*(6′)-*Ii* gene,[1] which encodes aminoglycoside acetyltransferase AAC(6′) that confers resistance to the synergy of the association of penicillin with tobramycin. Nevertheless, similar genes have not been previously detected in other enterococcal species. *Enterococcus hirae* and *Enterococcus durans* are frequently found in the intestine of animals and less frequently in humans,[2] and are occasionally involved in severe human infections.[3]

The characterization of two novel *aac*(6′)-*Ii*-like genes, species-specific for *E. hirae* and *E. durans*, is reported in this study. In agreement with the nomenclature suggestion of Vanhoof *et al.*[4] for the *E. faecium* acetyltransferase gene [*aac*(6′)-*Ii*], we have named the new genes as *aac*(6′)-*Iih* for *E. hirae*, and *aac*(6′)-*Iid* for *E. durans*.

Eight *E. hirae* strains with seven different PFGE-*Sma*I patterns and three unrelated *E. durans* strains were identified by biochemical and genetic criteria.[5] MICs of penicillin, streptomycin, gentamicin, tobramycin and kanamycin were determined by the recommended agar dilution method, and none of the isolates had either high-level resistance to the aminoglycosides tested or penicillin resistance. Time–kill studies were carried out as previously described.[6] Synergy was observed with

the association of penicillin plus streptomycin or gentamicin in the eight *E. hirae* and the three *E. durans* strains. Absence of synergy occurred when penicillin was associated with either tobramycin or kanamycin.

Aminoglycoside acetyltransferase enzymes (AAC) were analysed in sonic extracts obtained by ultrasonic disruption, using the phosphocellulose paper-binding assay. A typical AAC(6′) activity was observed by the radioenzymic assay in all five of eight *E. hirae* and three of three *E. durans* strains tested. Enzymic modification of gentamicin C1a, tobramycin and netilmicin but not of gentamicin C1 and 6′-*N*-ethylnetilmicin was observed in all these strains. Phosphotransferase or nucleotidyltransferase activities were not observed in any strain.

For PCR assays, degenerate primers *aac* D-F (5′ → 3′) TGGGARYTICAYCCIHTIGT and *aac* D-R (5′ → 3′) YWWICCRTTIGCRTTIGGIAD were designed comparing the sequences of the different *aac*(6′)-*I* genes. Total DNAs from three *E. hirae* strains and one *E. durans* strain were used as templates obtaining a 270 bp amplicon in all cases. The nucleotide sequences were identical in the three *E. hirae*, but different in the *E. durans* strain, even though both PCR products showed high homology with *aac*(6′)-*Ii* from *E. faecium*. A new set of primers, *hira*-3 (5′ → 3′) CTTTGCAAAGTTACAAGAA and *hira*-4 (3′ → 5′) CTGTCCCTAGATAGATGA, were applied in an inverse PCR strategy using the total DNA generated from *E. hirae* AR48 strain digested with *Rsa* I and subsequent re-ligation, yielding a 0.8 kb amplicon. The primers *aac*1 (5′ → 3′) GGATAGCGGATGATTATCA and *aac*2 (3′ → 5′) TAAGAGTTTAATGAATAATTA were designed to amplify a fragment of ~840 bp containing the entire *aac*(6′) gene that has a size of 549 bp. The nucleotide sequence of the *aac*(6′)-*Iih* gene (G + C = 43.2%) from *E. hirae* strain presented a 72% identity with *aac*(6′)-*Ii* from *E. faecium* (G + C = 38.9%). The *aac*(6′)-*Iid* gene from *E. durans* (G + C = 42.8%) was obtained using the same strategy as for *E. hirae*, and showed 80% identity with *aac*(6′)-*Ii* from *E. faecium* and 73% identity with *aac*(6′)-*Iih* from *E. hirae*. The nucleotide sequences of the *aac*(6′)-*Iih* and *aac*(6′)-*Iid* genes have been deposited in the EMBL Nucleotide Sequence Database under the accession numbers AJ584700 and AJ584701, respectively.

The deduced amino acid sequences for AAC(6′)-Iih and AAC(6′)-Iid are compared with that of AAC(6′)-Ii in Figure 1. Sequence homologies were 65% and 68% between *E. faecium* and *E. hirae* or *E. durans*, respectively, and 76% between *E. durans* and *E. hirae*. All three motifs involved in the antibiotic binding domain were highly conserved in the three enzymes: the hydrophobic region located in position 70 to 77 (GWELHPLV), the region between amino acids 86 and 98 that corresponded to the sequence $Q^{I}/_{V}GTRLV^{S}/_{N}YLEKE$, and the region from amino acid 147 to 154 ($^{E}/_{T}$FYEKLGY).

Dot-blot hybridization assays were performed to evaluate the species-specificity of *aac*(6′)-*Iih* and *aac*(6′)-*Iid*, using total DNA from *E. faecalis* ($n = 10$), *E. faecium* ($n = 10$), *E. durans* ($n = 3$) and *E. hirae* ($n = 8$) isolates. Internal fragments of 270 bp obtained from *E. hirae* AR25, *E. durans* AR23 and *E. faecium* RC714 strains were used as templates. Positive hybridization was observed in the *E. faecium*, *E. durans* or *E. hirae* strains when the correspondent probes obtained from each species were applied; however, no cross-hybridization between the different species was observed. Negative amplifications were consistently obtained when total DNA from *E. faecalis* JH2-2 and ATCC 29212 strains was tested. This suggests that the AAC(6′)-Ii-like proteins might have originated very early in a common enterococcal ancestor, and then were later deleted in *E. faecalis* evolution.

In summary, new *aac*(6′)-*Ii*-like genes have been characterized in *E. hirae* and *E. durans*. The presence of these genes precludes the synergy between tobramycin or kanamycin/amikacin, and β-lactams, even though the strains can be categorized as non-high-level-resistant to aminoglycosides in conventional susceptibility testing studies. These results indicate that, except for the case of *E. faecalis*, association of β-lactams with tobramycin and kanamycin/amikacin should not be recommended in the therapy of enterococcal infections.

Acknowledgements

Part of this work was presented at the 12th ESCMID meeting (Milan 2002) and at the 43rd ICAAC meeting (Chicago 2003). This work was supported in part by the Red Temática REIPI C03/14 from the Instituto Carlos III and by grant FIS 00/545 from the Ministerio de Sanidad from Spain.

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AAC(6´)-Ii   MIISEFDRNNPVLKDQLSDLLRLTWPEEYGDSSAEEVEEMMNPERIAVAAVDQDELVGFIG
AAC(6´)-Iid          D L  R   A        D   EQPMK   RLLED     S IEG   I  V
AAC(6´)-Iih          E I  R   A        D   TEPMK   QL A      S IEGE     V

AAC(6´)-Ii   AIPQYGITGWELHPLVVESSRRKNQIGTRLVNYLEKEVASRGGITIYLGTDDLDHGTTL-S
AAC(6´)-Iid        Q            MS  Q V     S     I  Q   VV      VEGQ S AI
AAC(6´)-Iih        K          A TH  Q       S        Y  LV       VEGQ N -V

AAC(6´)-Ii   QTDLYVHTFDKVASIQNLREHPYEFYEKLGYKIVGVLPNANGWDKPDIWMAKTIIPRPDSQ
AAC(6´)-Iid  EE  FED    LET   RKD           Q      D    N         R ARKHG E
AAC(6´)-Iih  E   FED  A LQE K INH   T       Q I  I D    NQ    L  RVAK EPTE
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Figure 1. Amino acid sequences of AAC(6′)-Ii from *E. faecium*, AAC(6′)-Iid from *E. durans* and AAC(6′)-Iih from *E. hirae*.

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Journal of Antimicrobial Chemotherapy
doi:10.1093/jac/dki115
Advance Access publication 11 April 2005

Heteroresistance to carbapenems in *Acinetobacter baumannii*

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Keywords: imipenem, meropenem, Etest, PFGE, resistance, heterogeneity

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Sir,

Acinetobacter baumannii is an opportunistic pathogen with increasing relevance in hospital infections. The organism is particularly associated with nosocomial invasion of burn wounds, pneumonia, bacteraemia, post-neurosurgical meningitis and infections of the urinary tract. Recent data suggest that in several hospital settings large percentages of *A. baumannii* isolates are resistant to carbapenems.[1] Thus, treatment of carbapenem-resistant *A. baumannii* has become a clinical challenge, since this microorganism is usually resistant to almost all available antimicrobial agents.

Resistance to carbapenems among acinetobacters as well as among other bacteria is known to be homogeneous within a culture. In a previous study,[2] as is typical for β-lactam antibiotics, heteroresistance to meropenem has been detected in population studies among methicillin-resistant staphylococci. The present report describes the spread of hetero-carbapenem-resistant *A. baumannii* isolates.

In our region, carbapenem-resistant *A. baumannii* are being isolated with increasing frequency from clinical sources.[3] Moreover, several Gram-negative species producing VIM-type metallo-β-lactamases have been detected in Greek hospitals since 1997. Therefore, clinical isolates of *A. baumannii* are routinely screened by Etest (AB Biodisk, Solna, Sweden) for imipenem and meropenem, in addition to automated or disc diffusion susceptibility testing, in order to detect carbapenem-resistant isolates. Mueller–Hinton agar plates are inoculated with 0.5 McFarland standard suspension test organisms and the relevant Etest strips are added. Plates are incubated at 37°C for 18 h. During this procedure, eight clinical isolates of *A. baumannii* that showed subcolonies present in the clear zone of inhibition were recovered from separate patients. The isolates exhibited resistance to all other available antimicrobials except colistin; three of them were recovered from urine specimens, three from bronchial secretions and two from blood specimens. They were provisionally identified to genus species by the API 20NE system (bioMérieux API, Marcy l'Étoile, France) according to the manufacturer's instructions. The identification of *A. baumannii* was performed by a simplified identification scheme.

Imipenem and meropenem MIC determinations for the eight acinetobacters were repeated by Etest according to the manufacturer's specification; their MICs were in the range 3–12 mg/L and 2–8 mg/L, respectively. However, when the Etest MICs took into account colonies growing within the apparent inner zone of inhibition a subpopulation of resistant cells was observed in the Etest zone of inhibition with an imipenem or meropenem MIC of $\geqslant$32 mg/L (Figure 1). The same results were obtained when the assay was repeated by using plastic strips from a fresh cartridge and by testing a susceptible control organism in tandem. When the resistant colonies were re-tested the imipenem and meropenem MICs remained in the above ranges and again a subpopulation of resistant isolates was grown in the zone of inhibition. Resistant subpopulations were also grown within the zone of inhibition around imipenem and meropenem discs in the disc diffusion test (data not shown).

The MICs of imipenem and meropenem were determined by the agar dilution method according to the NCCLS guidelines.[4] The assay was performed by inoculating 10^4 cfu/spot onto cation-supplemented Mueller–Hinton agar plates containing antibiotic dilutions in the range 0.12–512 mg/L. The MICs for both the original isolates and the subcolonies from within the Etest

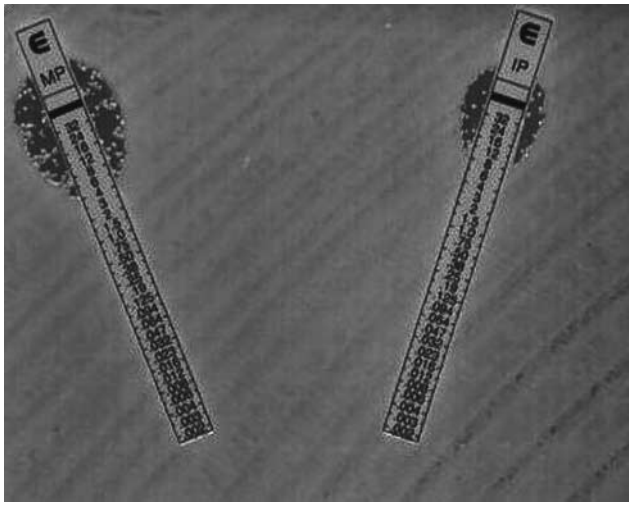

Figure 1. Imipenem and meropenem Etests on *A. baumannii* showing resistant subpopulation. In this particular instance, imipenem and meropenem MICs were 12 and 6 mg/L, respectively, but a subpopulation of resistant cells was grown at up to an imipenem or meropenem concentration of $>$ 32mg/L.