The Journal of Antibiotics (2017) 70, 400–403

www.nature.com/ja

ORIGINAL ARTICLE

New aminoglycoside-modifying enzymes APH(3′)-VIII and APH(3′)-IX in *Acinetobacter rudis* and *Acinetobacter gerneri*

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Analysis of whole-genome sequences of 133 strains of *Acinetobacter* detected two genes for new types of aminoglycoside 3′-*O*-phosphotransferase [APH(3′)], type VIII in *Acinetobacter rudis* and IX in *A. gerneri*. The enzymes were related to each other (49% identity) and to APH(3′)-VI (61% and 51% identity, respectively), which is intrinsic to *A. guillouiae*. The cloned genes conferred kanamycin and amikacin resistance to *Escherichia coli* but were cryptic or expressed at low levels in the original hosts. The chromosomal location of both genes and the genetic events for acquisition of an ancestral *aphA* gene by *A. rudis* and *A. gerneri*, and loss by *A. bereziniae* were supported by the molecular phylogenetic tree of these genes. These data confirm that nonpathogenic susceptible bacterial species can be considered as potential reservoirs of resistance genes.
The Journal of Antibiotics (2017) **70,** 400–403; doi:10.1038/ja.2016.144; published online 14 December 2016

INTRODUCTION

Acinetobacter spp., especially *Acinetobacter baumannii*, are associated with epidemics of hospital-acquired infections, in particular in intensive care units.[1] The infections are difficult to cure as they occur in immunocompromised patients and because these opportunistic pathogens, notably *A. baumannii*, have accumulated multidrug resistance mechanisms.[1] In addition to broad intrinsic resistance, acquired resistance is the result of horizontal gene transfer[2–4] or of mutations affecting expression of efflux genes.[5]

Aminoglycosides are, with β-lactams, the most useful drug classes for the treatment of infections due to *Acinetobacter*. Although resistance to aminoglycosides due to 16S ribosomal RNA methylation, in particular by ArmA, is increasingly reported,[6] the most common mechanism remains enzymatic inactivation of the drugs, primarily by 6′-*N*-acetyltransferases [AAC(6′)] and 3′-*O*-phosphotransferases [APH(3′)].

We have analysed the genomes of 133 strains of *Acinetobacter* spp. covering the breadth of the known taxonomic diversity of the genus, which allowed to build a robust phylogeny of the entire genus.[7] This new evolutionary tree was used to assess pending taxonomic issues and sample the genus for key mechanisms generating genetic variability.

We have also systematically screened these genomes for the presence of genes encoding aminoglycoside-modifying enzymes. Comparative analysis of the various types (isozyme forms) and subtypes (resistance profile conferred) of APH(3′) enzymes in *Acinetobacter* allowed us to distinguish the known types and subtypes and also to identify new types.[8] We recently reported the origin of aminoglycoside 3′-*O*-phosphotransferase type VI [APH(3′)-VI] in the chromosome of *A. guillouiae*,[8] an environmental species distantly related to *A. baumannii* and rarely responsible for human infections and its intra- and inter-generic dissemination.

In the present study, we document two new types of APH(3′) enzymes, type VIII in *A. rudis* and type IX in *A. gerneri*, two species that have been isolated only from diverse environmental sources.[9]

MATERIALS AND METHODS

Bacterial strains

The whole-genome sequences of 133 strains of *Acinetobacter*[7] were studied. Two additional strains of *A. rudis* were screened for the presence of the *aphA* gene and were identified as *A. rudis* based on comprehensive phenotypic testing, *rpoB* gene comparative analysis and whole-cell MALDI-TOF MS profiling (Nemec and Krizova, unpublished data). *Escherichia coli* One Shot TOP10 (Invitrogen, San Diego, CA, USA) was used as a recipient for cloning the *aphA* genes. Bacteria were grown, according to their physiological requirements, at 30 –37 °C, in brain heart infusion broth and agar (Difco Laboratory, Detroit, MI, USA).

Antimicrobial susceptibility testing

MICs were determined by agar dilution in cation-adjusted Mueller-Hinton (MH) agar according to the CLSI guideline.[10]

Sequence analysis

The *aphA* genes were inferred from whole-genome sequences obtained previously.[7] Multiple sequence alignments and the amino acid identity calculation of the deduced protein sequences were carried out using the MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/).

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Received 12 August 2016; revised 3 October 2016; accepted 31 October 2016; published online 14 December 2016

Table 1 Percent identity and similarity between ancestral APH(3′) types in *Acinetobacter* spp.

% identity[a]			*% similarity*[a]		*VIII*			
Type	*Accession no.*	*Species*	*Strain*[b]	*VI*	*CIP 110305*^{T}	*ANC 4156*	*ANC 4130*	*IX*
VI	ENV16316	*A. guillouiae*	**NIPH 991**		73.3	73.3	73.3	64.7
VIII	EPF73263	*A. rudis*	**CIP 110305**^{T,b,c}	**60.5**		100	99.6	64.3
			ANC 4156	**60.5**	**100**		99.6	64.3
			ANC 4130[c]	**60.5**	**99.6**	**99.6**		63.9
IX	ENV34035	*A. gerneri*	**CIP 107464**^{T}	**50.8**	**49.2**	**49.2**	**48.9**	

Abbreviation: T, type strain.
[a]Identity (bold underlined) and similarity (underlined).
[b]Strains with a whole genome sequenced are indicated in bold face.
[c]Strains from Vaz-Moreira *et al.*[8]

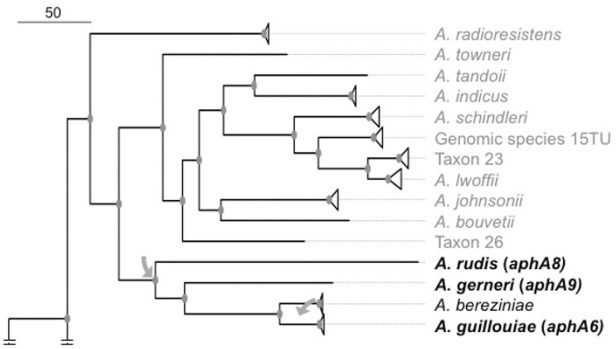

Figure 1 Phylogeny of the *Acinetobacter* genus based on the alignment of the protein families of the core genome. Triangles mark groups of taxa that are from the same species or have more than 95% average nucleotide identity values and, therefore, might be regarded as coming from the same species. The nodes in red have bootstrap support higher than 95%. The tree was rooted using two outgroup genomes. Curved arrows, acquisition or loss of *aphA* gene. Modified from Yoon *et al.*[7] A full color version of this figure is available at *The Journal of Antibiotics* journal online.

DNA manipulation and recombinant DNA techniques

Genomic DNA was extracted by boiling as described.[8] DNA amplification was performed in a GeneAmp PCR system 9700 (Perkin Elmer Cetus, Norwalk, CT, USA) with Phusion high-fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA). The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA, USA). Digestion with restriction endonucleases (New England Biolabs, Ipswich, MA, USA), ligation with T4 DNA ligase (New England Biolabs) and transformation with recombinant plasmid DNA were performed by standard methods. Plasmid DNA was purified with a Nucleospin plasmid miniprep kit (Macherey-Nagel GmbH & Co., Düren, Germany). Nucleotide sequencing was carried out with a CEQ 8000 DNA analysis system automatic sequencer (Beckman Instrument, Inc., Palo Alto, CA, USA).

Identification of the APH(3′) enzymes

The putative homologs between pairs of genomes were determined with BlastP[11] based on the sequence of APH(3′)-VI (GenBank Accession ID, P09885).[12] The presence of *aphA8* in the additional *A. rudis* strains was searched for by PCR using primers aphA8-F, 5′-ATGAAACTACCTCAGAA AAT-3′ and aphA8-R, 5′-ATTCAATTTAACTCATCAAGTTT T-3′.

Cloning of *aphA* genes

The 808-bp and 829-bp fragments including the ribosomal binding site (RBS) of phage T7 gene was amplified from *A. rudis* CIP 110305^T and from *A. gerneri* CIP 107464^T with primers aphA8-RBS-F, 5′-tttaagaaggagatatacatATGAAAC TACCTCAGAAAAT-3′ and aphA8-R, and aphA9-RBS-F, 5′-tttaagaaggagata tacatATGATCAATGATATGAAAAT-3′ and aphA9-R, 5′-GATCAATTTAACT CATCCAATT-3′, respectively (RBS in lower case, start codon underlined). The amplicons were ligated to the pCR-Blunt vector (Invitrogen), the recombinant plasmids digested with *Bam*HI and *Xba*I, the inserts ligated to *Bam*HI-*Xba*I-linearized pUC18 and transformed into *E. coli* TOP10 with selection on ampicillin 100 μg ml^{-1} and kanamycin 8 μg ml^{-1}. The orientation and sequence of all the inserts were verified with forward and reverse universal primers.

RESULTS AND DISCUSSION

Analysis of the whole-genome sequences of 133 strains of *Acinetobacter*[7] allowed to detect two new types of APH(3′) enzymes, type VIII (GenBank accession ID, EPF73263) in *A. rudis* CIP 110305^T and type IX (GenBank accession ID, ENV34035) in *A. gerneri* CIP 107464^T with, respectively, 61% and 51% sequence identity relative to APH(3′)-VI from *A. guillouiae* NIPH 991 (GenBank accession ID, ENV16316) (Table 1), which is intrinsic to this species.[8] The identity between APH(3′)-VIII and -IX proteins was 49%. The mol% G+C content of the corresponding structural genes *aphA8* and *aphA9* was 34% and 33%, respectively, close to those of the host species (40 and 39%) suggesting, as for *aphA6*,[8] an origin in *Acinetobacter* for the genes. No other aminoglycoside resistance genes were detected in these strains.

In the phylogeny of the *Acinetobacter* genus,[7] *A. rudis* (*aphA8*) and *A. gerneri* (*aphA9*) constitute a clade together with *A. guillouiae* (*aphA6*) and *A. bereziniae* devoid of *aphA* gene (Figure 1). As *A. rudis* and *A. gerneri* are early branches, this is consistent with acquisition of an ancestral *aphA* gene at the split with the neighboring clade and loss by *A. bereziniae*. The chromosomal location of *aphA8* and *aphA9* and the genetic events for acquisition and loss of the *aphA* genes were also supported by the molecular phylogeny of these genes and of *aphA6*.[8]

In *A. rudis* CIP 110305^T and DSM24031 (GenBank RefSeq: NZ_BBRX01000095), the *aphA8* gene was located in an 11.5-kb contig. Two additional *A. rudis* strains, ANC 4156 and ANC 4130, were screened for the presence of *aphA8* by PCR and were found to harbor the gene, which supports the notion that it is intrinsic to this species. In *A. gerneri* CIP 107464^T, *aphA9* was located in a 201-kb contig that also carried the gene for ribosomal protein L31 confirming a chromosomal location of *aphA9*. *A. rudis* and *A. gerneri* are rarely isolated and we could not screen for the presence of the genes in more strains.

The MICs of neomycin B and amikacin against the three strains were similarly low (Table 2). *A. gerneri* CIP 107464^T was also susceptible to both aminoglycosides (Table 2).

The *aphA* genes from *A. rudis* CIP 110305^T and *A. gerneri* CIP 107464^T, cryptic or expressed at low levels in the original hosts, were expressed and functional after cloning under the control of the P_{lac}

Table 2 Susceptibility to aminoglycosides

		MIC (μg ml^{-1})[a]			
Strain/plasmid	*APH(3′) type*	*Kanamycin*	*Neomycin B*	*Amikacin*	*Tobramycin*
E. coli TOP10		2	2	2	0.5
E. coli TOP10/pUC18		2	2	2	0.5
***A. rudis* CIP 110305^{T,b,c}**	**VIII**	**16**	**4**	**4**	**0.5**
A. rudis ANC 4156	VIII	2	4	4	0.5
A. rudis ANC 4130[c]	VIII	4	4	2	0.5
E. coli TOP10/pUC18Ω*aphA8*	VIII	64	8	8	0.5
***A. gerneri* CIP 107464^{T}**	**IX**	**4**	**1**	**1**	**0.25**
E. coli TOP10/pUC18Ω*aphA9*	IX	128	16	16	0.5

Abbreviation: T, type strain.
[a]MICs were determined by agar dilution.[10]
[b]Strains with a whole genome sequenced are indicated in bold face.
[c]Strains from Vaz-Moreira *et al.*[8]

promoter into *E. coli* TOP10 where they conferred resistance to substrate aminoglycosides (Table 2). As for other APH(3′) enzymes,[8] the MICs of kanamycin were higher than those of amikacin (Table 2).

As we were unable to detect identical or closely related proteins in species other than *A. rudis* and *A. gerneri* in our strain collection[7] and in the GenBank database, it is likely that dissemination of the corresponding genes did not occur within or outside the *Acinetobacter* genus. This is in contrast with the *aac(6′)-Ih* gene originating in *A. gyllenbergii,* which has disseminated in the *Acinetobacter* genus (JAC 2016).

APH(3′) enzymes are largely spread in Gram-negative and -positive bacteria. They display homology with eukaryotic protein kinases,[12,13] probably as a consequence of an adaptative process during evolution. It has also been shown that APHs can phosphorylate several eukaryotic protein kinase substrates on serine residues.[14] In addition, APH enzymes are able to catalyze hydrolysis of ATP;[15,16] as these enzymes are produced constitutively and the turnover of ATP leads to a fitness cost.[17] Phosphorylation involves enzymatic transfer of the γ–phosphate of ATP to an hydroxyl group. The 3′ hydroxyl group of kanamycin B is missing in tobramycin (3′-deoxykanamycin B), which accounts for lack of resistance to tobramycin (Table 2). Seven APH(3′) subtypes have been reported on the basis of their substrate recognition.[8] APH(3′)-I and -II of Gram-negative bacteria have no clinical consequences as neomycin and kanamycin are not used any more in therapy of systemic infections. In contrast, APH(3′)-III of Gram-positive cocci, APH(3′)-VI common in *Acinetobacter* and rare in enterobacteria and *Pseudomonas* and, at a lesser extent, APH(3′)-VII in *Campylobacter* all affect the activity of amikacin and are thus clinically relevant. APH(3′)-IV and -V are confined to the aminoglycoside-producing microorganisms.

Like APH(3′)-IIb from *Pseudomonas aeruginosa,*[18] -IIc from *Stenotrophomonas maltophilia*[19] and -VI from *A. guillouiae*,[8] respectively, APH(3′)-VIII and -IX are new, putatively species specific, enzymes. *A. rudis* and *A. gerneri* could, therefore, represent additional examples of environmental species that act as reservoirs of resistance genes.

The notion that aminoglycoside-modifying enzymes originate from antibiotic producers is based on horizontal gene transfer between soil microorganisms and pathogenic bacteria.[20] However, in other instances, and as discussed above, a physiological role is consistent with *aphA* being housekeeping genes.[21] Our data confirm that resistance genes can also be found in susceptible environmental bacteria.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank A Nemec for the gift of *A. rudis* strains and T Lambert for helpful discussions. E-JY was supported by an unrestricted grant from Reckitt-Benckiser. This work is dedicated to Professor Julian Davies in whose laboratory PC was a research associate from 1974 to 1977 in the Department of Biochemistry at the University of Wisconsin-Madison. Julian was very stimulating, suggesting all sorts of new research projects, collaborations and advanced techniques. He was a pioneer in the study of the origin of antibiotic resistance genes and of the most clinically important resistance mechanisms in human bacterial pathogens.

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