RESEARCH ARTICLE

Origin in *Acinetobacter guillouiae* and Dissemination of the Aminoglycoside-Modifying Enzyme Aph(3′)-VI

Eun-Jeong Yoon,[a] Sylvie Goussard,[a] Marie Touchon,[b,c] Lenka Krizova,[d] Gustavo Cerqueira,[e] Cheryl Murphy,[e] Thierry Lambert,[f] Catherine Grillot-Courvalin,[a] Alexandr Nemec,[d] Patrice Courvalin[a]

Institut Pasteur, Unité des Agents Antibactériens, Paris, France[a]; Institut Pasteur, Microbial Evolutionary Genomics, Paris, France[b]; CNRS, UMR 3525, Paris, France[c]; Laboratory of Bacterial Genetics, National Institute of Public Health, Prague, Czech Republic[d]; Broad Institute of Harvard and MIT, Cambridge, Massachusetts, USA[e]; EA4043, Faculté de Pharmacie, Université Paris Sud, Châtenay-Malabry, France[f]

ABSTRACT **The amikacin resistance gene *aphA6* was first detected in the nosocomial pathogen *Acinetobacter baumannii* and subsequently in other genera. Analysis of 133 whole-genome sequences covering the taxonomic diversity of *Acinetobacter* spp. detected *aphA6* in the chromosome of 2 isolates of *A. guillouiae*, which is an environmental species, 1 of 8 *A. parvus* isolates, and 5 of 34 *A. baumannii* isolates. The gene was also present in 29 out of 36 *A. guillouiae* isolates screened by PCR, indicating that it is ancestral to this species. The P_{native} promoter for *aphA6* in *A. guillouiae* and *A. parvus* was replaced in *A. baumannii* by P_{aphA6}, which was generated by use of the insertion sequence IS*Aba125*, which brought a −35 sequence. Study of promoter strength in *Escherichia coli* and *A. baumannii* indicated that P_{aphA6} was four times more potent than P_{native}. There was a good correlation between aminoglycoside MICs and *aphA6* transcription in *A. guillouiae* isolates that remained susceptible to amikacin. The marked topology differences of the phylogenetic trees of *aphA6* and of the hosts strongly support its recent direct transfer within *Acinetobacter* spp. and also to evolutionarily remote bacterial genera. Concomitant expression of *aphA6* must have occurred because, contrary to the donors, it can confer resistance to the new hosts. Mobilization and expression of *aphA6* via composite transposons and the upstream IS-generating hybrid P_{aphA6}, followed by conjugation, seems the most plausible mechanism. This is in agreement with the observation that, in the recipients, *aphA6* is carried by conjugative plasmids and flanked by IS that are common in *Acinetobacter* spp. Our data indicate that resistance genes can also be found in susceptible environmental bacteria.**

IMPORTANCE **We speculated that the *aphA6* gene for an enzyme that confers resistance to amikacin, the most active aminoglycoside for the treatment of nosocomial infections due to *Acinetobacter* spp., originated in this genus before disseminating to phylogenetically distant genera pathogenic for humans. Using a combination of whole-genome sequencing of a collection of *Acinetobacter* spp. covering the breadth of the known taxonomic diversity of the genus, gene cloning, detailed promoter analysis, study of heterologous gene expression, and comparative analysis of the phylogenetic trees of *aphA6* and of the bacterial hosts, we found that *aphA6* originated in *Acinetobacter guillouiae*, an amikacin-susceptible environmental species. The gene conferred, upon mobilization, high-level resistance to the new hosts. This work stresses that nonpathogenic bacteria can act as reservoirs of resistance determinants, and it provides an example of the use of a genomic library to study the origin and dissemination of an antibiotic resistance gene to human pathogens.**

Received 17 September 2014 **Accepted** 22 September 2014 **Published** 21 October 2014

Citation Yoon E, Goussard S, Touchon M, Krizova L, Cerqueira G, Murphy C, Lambert T, Grillot-Courvalin C, Nemec A, Courvalin P. 2014. Origin in *Acinetobacter guillouiae* and dissemination of the aminoglycoside-modifying enzyme Aph(3′)-VI. mBio 5(5):e01972-14. doi:10.1128/mBio.01972-14.

Editor Julian E. Davies, University of British Columbia

Address correspondence to Patrice Courvalin, patrice.courvalin@pasteur.fr.

This article is a direct contribution from a Fellow of the American Academy of Microbiology.

Acinetobacter spp. are increasingly responsible for severe nosocomial infections, in particular, in intensive care units (1). *Acinetobacter* ventilator-associated pneumonia, urinary tract infections, and bacteremia are difficult to cure, at least in part because strains are often resistant to multiple antimicrobial agents. Although *Acinetobacter baumannii* is the most prevalent pathogen in the genus, other species have been shown to be responsible for human infections, such as *Acinetobacter pittii* and *Acinetobacter nosocomialis* and, to a lesser extent, *Acinetobacter ursingii*, *Acinetobacter haemolyticus*, *Acinetobacter lwoffii*, and *Acinetobacter parvus* (2). Improvement of identification will likely indicate that additional *Acinetobacter* spp. can also be isolated in clinical settings.

In *A. baumannii*, chromosomally encoded β-lactamases, basal expression of efflux pumps, and low membrane permeability are responsible for broad intrinsic resistance. This species also has the remarkable ability to become resistant to numerous antibiotics by horizontal acquisition of mobile genetic elements, plasmids (3), transposons (4), resistance islands (5), or a combination of all (6).

Aminoglycosides, together with β-lactams and quinolones, are the major drug classes used in therapy of *Acinetobacter* infections.

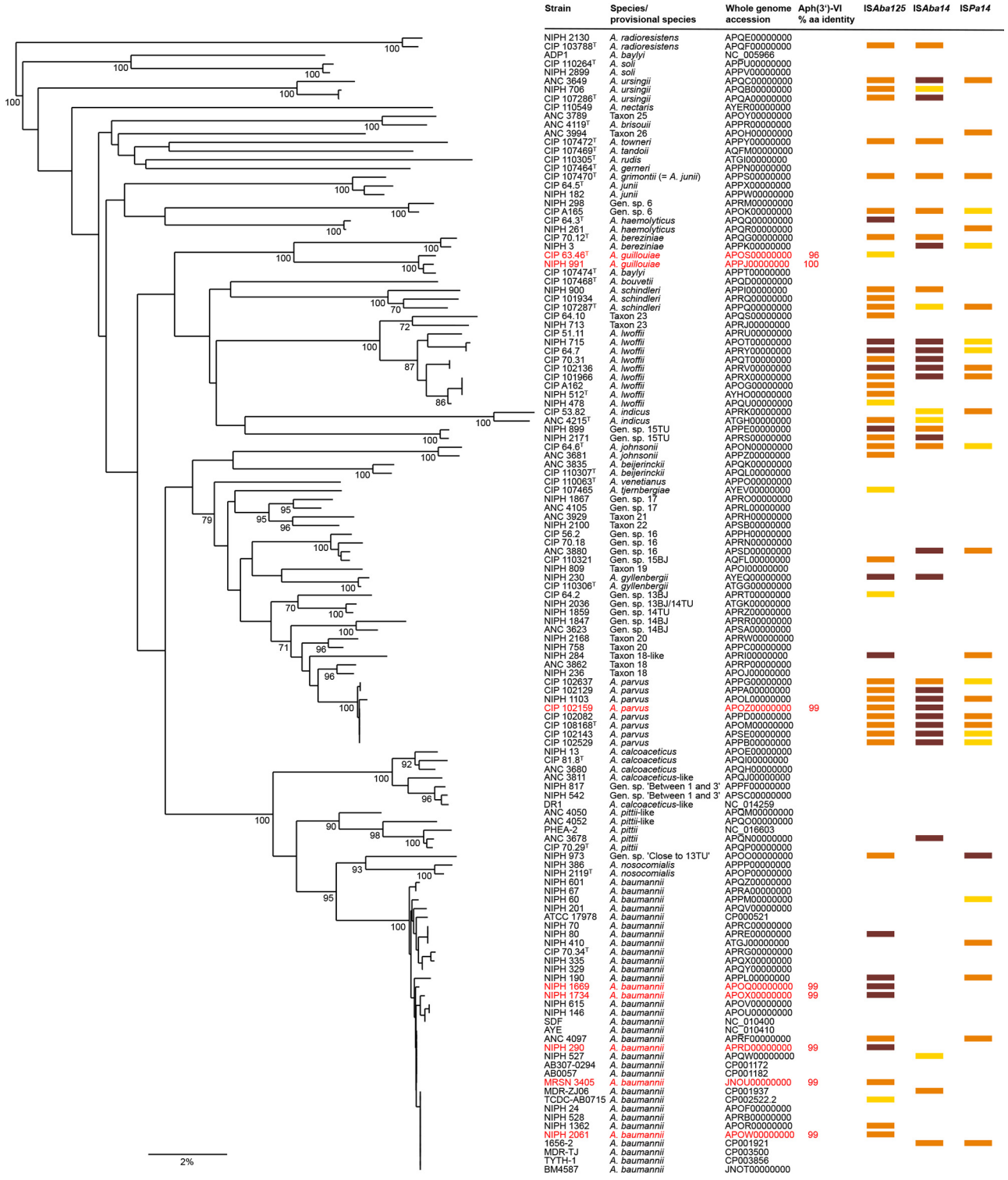

FIG 1 Neighbor-joining tree based on a partial (861-bp) sequence of the *rpoB* gene. Bootstrap values (≥70%) obtained after 1,000 replicates are given at the nodes. Bar, 2% sequence divergence. The percent amino acid identity is relative to *A. guillouiae* NIPH 991. The sequences were compared using the neighbor-joining method and a simple matching cost matrix. The accession numbers of the genome sequences from which the data were retrieved are indicated in the third column. Red, genome with *aphA6*. The distribution of the insertion sequences indicated at the top is represented with a color code: white, absence of IS; yellow, single copy; orange, a few (2 to 9) copies; brown, numerous (≥10) copies. The high similarity of the *rpoB* sequences of *A. guillouiae* and *A. baylyi* CIP 107474^T is likely a result of the transfer of the complete *rpoB* gene from *A. guillouiae* to this *A. baylyi* strain (31).

TABLE 1 Similarities between homologs and Aph(3′)-VI proteins

Species and strain	% similarity with homolog or Aph(3′)-VI protein[a]							
	A. guillouiae		*A. parvus*	*A. baumannii*				
	CIP 63.46^T	NIPH 991	CIP 102159	NIPH 1669	NIPH 1734	NIPH 2061	NIPH 290	MRSN 3405
A. guillouiae								
CIP 63.46^T		98.8	89.5	89.1	89.1	89.1	89.2	89.2
NIPH 991	**97.4**		89.2	89.1	89.1	89.1	89.1	89.0
A. parvus								
CIP 102159	**98.1**	**99.3**		90.6	90.6	90.6	90.6	90.6
A. baumannii								
NIPH 1669	**97.6**	**99.2**	**99.5**		99.3	99.3	99.4	99.4
NIPH 1734	**97.6**	**99.2**	**99.5**	**100.0**		99.3	99.3	99.3
NIPH 2061	**97.6**	**99.2**	**99.5**	**100.0**	**100.0**		99.3	99.3
NIPH 290	**97.6**	**99.2**	**99.5**	**100.0**	**100.0**	**100.0**		99.8
MRSN 3405	**98.1**	**99.3**	**100.0**	**99.7**	**99.7**	**99.7**	**99.7**	

[a] The average similarities between homologous proteins (values in lightface) or with Aph(3′)-VI (values in boldface); similarity was defined as unique pairwise reciprocal best hits with at least 80% similarity in amino acid sequence and less than 20% difference in protein length. Analysis of orthology was made for every pair of genomes.

Despite side effects, ototoxicity and nephrotoxicity, and increasing resistance, the aminoglycosides retain therapeutic value, in particular because of their bactericidal activity and the synergy they display with β-lactams.

There are four known mechanisms of resistance to aminoglycosides in bacterial human pathogens: (i) decreased intracellular accumulation of the antibiotic (7); (ii) modification of the target (8); (iii) trapping of the drug (9); (iv) enzymatic modification of the drug (10), primarily through N-acetylation, O-nucleotidylation, or O-phosphorylation, which is the most common mechanism. Since amikacin, because of an (*S*)-4-amino-2-hydroxybutyrate side chain moiety substituted at the N-1 position of the 2-deoxystreptamine ring, is inactivated in Gram-negative bacteria only by 6′-acetyltransferases type I and 3′-phosphotransferases types VI and VII (10), it remains one of the most active aminoglycosides for the treatment of infections due to *Acinetobacter* spp. (11).

Aminoglycoside 3′-phosphotransferase type VI [Aph(3′)-VI] was first reported in *A. baumannii*, and the structural gene for the enzyme, *aphA6*, was carried by self-transferable plasmids (12). Subsequent dissemination of this resistance determinant in various members of the family *Enterobacteriaceae* and in *Pseudomonas aeruginosa* is due to dissemination of the 5.2-kb Tn*1528* composite transposon delimited by two copies of IS*15*-Δ (13). More recently, it has been reported that, in *A. baumannii*, *aphA6* is also part of the transposon Tn*aphA6*, in which it is immediately flanked by two copies of IS*Aba125* (14). The low G+C content of *aphA6*, 33 mol%, and the link with an insertion sequence widely spread among *Acinetobacter* spp. suggest an origin in this genus for the gene.

We have analyzed the genomes of 133 strains of *Acinetobacter* spp., covering the breadth of the known taxonomic diversity of the genus, which allowed to us to build a robust phylogeny of the entire genus (M. Touchon, J. Cury, E. J. Yoon, L. Krizova, G. Cerqueira, C. Murphy, M. Feldgarden, J. Wortman, D. Clermont, T. Lambert, C. Grillot-Courvalin, A. Nemec, P. Courvalin, E. P. Rocha, submitted for publication). This new evolutionary tree was used to assess pending taxonomic issues and sample the genus for key mechanisms generating genetic variability, and it allowed the detection of genes for numerous class D β-lactamases and *Acinetobacter*-derived cephalosporinases (15).

We have systematically screened these genomes for the presence of genes encoding aminoglycoside-modifying enzymes, and we report the origin of *aphA6* in the chromosome of *Acinetobacter guillouiae*, an environmental species distantly related to *A. baumannii* and rarely responsible for human infections, and this gene's intra- and intergenus dissemination.

RESULTS

The *aphA6* gene is intrinsic to *A. guillouiae*. Analysis of the whole-genome sequences of 133 strains of *Acinetobacter* allowed us to detect the *aphA6* gene in 5 out of 34 *A. baumannii* strains, 1 of 8 *A. parvus* strains, and both *A. guillouiae* strains (Fig. 1). The level of sequence similarity between the deduced Aph(3′)-VI proteins of the various species was always higher than the average sequence similarity across all homologs shared by the two species (Table 1). As an example, the Aph(3′)-VI proteins of *A. guillouiae* NIPH 991 and *A. parvus* CIP 102159 had 99.3% sequence similarity, whereas their homologs had on average only 89.2% similarity. In both *A. guillouiae* strains, *aphA6* was located in large contigs that also carried genes for ribosomal proteins S1, S6, S18, S20, S21, and L9, indicating a chromosomal location for *aphA6*. Thirty-six additional *A. guillouiae* isolates obtained from a wide spectrum of ecosystems, including human clinical specimens as well as soil and water samples from various natural locations, were screened for the presence of *aphA6* by PCR, and 29 were found to harbor the gene (Table 2). The high prevalence of *aphA6* among these ecologically diverse populations of *A. guillouiae* (82%) can be taken as evidence that the gene is intrinsic to this species.

Genomic environment of *aphA6*. The *A. guillouiae* and *A. parvus* strains had a nearly identical 153-bp sequence upstream from *aphA6* that included the P_{native} promoter (Fig. 2). The promoter was located from bp 104 to bp 75 upstream from the start codon of *aphA6*, and the −35 and −10 sequences were separated by 17 bp. The *aphA6* gene in the five *A. baumannii* isolates was part of a 3.1-kb composite transposon, Tn*aphA6*, which consists of two 1.1-kb directly oriented copies of IS*Aba125* bracketing the *aphA6* gene, as described for multiply antibiotic-resistant *A. baumannii* clinical strains (14). The 93 bp between the end of the right inverted repeat (IRR) of the upstream IS*Aba125* copy and the beginning of *aphA6* in the five *A. baumannii* isolates were identical to those in the *A. guillouiae* and *A. parvus* strains (Fig. 2; see also

TABLE 2 *A. guillouiae* strains studied

Strain	Specimen, country,[a] yr of isolation	*aphA6*[b]	MIC (μg/ml)[c]			Reference
			Kanamycin	Amikacin	Gentamicin	
ANC 4134[d]	Meadow mud, CZ, 2011	+	>32	8	1	This study
NIPH 2408[d]	Raw milk, CZ	+	>32	0.5	0.5	31
NIPH 820	Unknown	+	32	4	0.5	31
NIPH 2127	Wound (human), SE, 1980	+	32	4	0.5	31
NIPH 682[d]	Blood (human), CZ, 1997	+	32	2	1	31
ANC 3935[d]	Forest soil, CZ, 2011	+	32	2	0.5	This study
ANC 4146[d]	Forest soil, CZ, 2011	+	32	2	0.5	This study
ANC 4181	Pond mud, CZ, 2011	+	16	2	0.5	This study
ANC 3814[d]	Freshwater sediment, CZ, 2009	+	16	1	0.5	This study
CIP 63.46^{T}[d,e]	**Sewage, before 1951**	**+**	**16**	**1**	**0.5**	31
ANC 4111[d]	Freshwater sediment, CZ, 2011	+	8	0.5	0.25	This study
ANC 4170	Creek mud, CZ, 2011	+	8	2	0.5	This study
NIPH 2272	Contact lens, SE, 1980	+	8	1	1	31
ANC 4125	Meadow mud, CZ, 2011	+	8	1	1	This study
ANC 3626[d]	Forest soil, CZ, 2007	+	8	1	0.5	31
ANC 4145	Forest soil, CZ, 2011	+	8	1	0.5	This study
ANC 3826	Freshwater sediment, CZ, 2009	+	8	1	0.5	This study
ANC 3827	Freshwater sediment, CZ, 2009	+	8	1	0.5	This study
NIPH 2169	Sputum (human), DK, 1988–1989	+	8	1	0.25	31
ANC 3812	Freshwater sediment, CZ, 2009	+	4	0.5	0.5	This study
ANC 3679[d]	Forest soil, CZ, 2008	+	4	1	0.5	This study
ANC 4136	Freshwater sediment, CZ, 2011	+	2	1	1	This study
ANC 4143	Freshwater sediment, CZ, 2011	+	2	1	1	This study
NIPH 2689	Activated sludge plant, AU, 1995	+	2	0.5	0.5	31
ANC 4133[d]	Creek mud, CZ, 2011	+	2	0.5	0.5	This study
NIPH 2529	Blood (human), NL, 1999	+	1	0.5	0.5	31
ANC 4258[d]	Creek mud, CZ, 2012	+	1	0.5	0.5	This study
NIPH 991[d,e]	**Ear swab (human), CZ, 1998**	**+**	**1**	**0.5**	**0.25**	31
NIPH 769	Barley field soil, UK, 1993–1994	+	0.5	0.5	0.5	31
ANC 4184	Creek mud, CZ, 2011	+	0.5	0.5	0.25	This study
ANC 4140[d,f]	Meadow mud, CZ, 2011	+	1	0.5	0.5	This study
NIPH 2273[d]	Urine (human), SE, 1980	−	0.5	0.5	0.5	31
NIPH 2525	Freshwater with sediment, DK, 1997	−	0.5	0.5	0.25	31
NIPH 2536	Eye (cat), NL, 2001	−	1	0.5	0.5	31
NIPH 2681	Feces (human), NL, 2000	−	1	0.5	0.5	31
ANC 3834[d]	Freshwater sediment, CZ, 2009	−	0.5	0.5	0.5	This study
ANC 3911[d]	Forest soil, CZ, 2010	−	0.5	0.5	0.5	This study
NIPH 2680	Feces (human), NL, 2000	−	0.5	0.25	0.25	31

[a] Country abbreviations: AU, Australia; CZ, Czech Republic; DK, Denmark; NL, The Netherlands; SE, Sweden; UK, United Kingdom.
[b] Presence (+) or absence (−) of the gene, detected by PCR.
[c] MICs were determined by agar dilution (18, 19).
[d] Strain analyzed by qRT-PCR; the promoter and *aphA6* gene were sequenced.
[e] Strains for which the whole genome was sequenced. Information for such strains is shown in boldface.
[f] Strain with truncated *aphA6* gene.

Table S1 in the supplemental material). Insertion of the left IS*Aba125* copy in *A. baumannii* disrupted the P_{native} promoter between the −35 and −10 sequences and brought a new −35 sequence, generating the strong P_{aphA6} hybrid promoter. This promoter has recently been described as P_{NDM-1} upstream from bla_{NDM-1} (16); this region has been shown convincingly to be the result of a fusion, which likely occurred in *A. baumannii*, between the promoter of *aphA6*, designated P_{aphA6}, the first 19 bp of *aphA6*, and the N-terminal portion of the bla_{NDM-1} gene (17).

The two *A. guillouiae* strains have a nearly identical 100-kb region of DNA (similar gene content and order) flanking the *aphA6* gene, except for the presence of a large prophage integrated 2.5 kb upstream from *aphA6* in *A. guillouiae* CIP 63.46^{T} (Fig. 2). The genomic context of the *aphA6* gene in the *A. guillouiae* strains was determined by PCR mapping over ca. 4.5 kb (see Fig. S1 in the supplemental material). The 12 strains studied had *aphA6* flanking regions indistinguishable from those in strains NIPH 991 and CIP 63.46^{T}. No large insertions or deletions in the noncoding regions were detected. Strains ANC 4133 and ANC 4140 had undergone DNA rearrangements, deletions, or large insertions downstream from the first open reading frame (ORF) on the right side of *aphA6*. The conserved environment of the gene in *A. guillouiae* is consistent with its ancestral integration in the chromosome of this species. In the seven *aphA6*-negative *A. guillouiae* strains, only the upstream ORF was found, indicating loss of the gene following various deletion events (see Fig. S1).

In *A. parvus* CIP 102159, *aphA6* was part of a small contig (ca. 1.8 kb) flanked by 53-bp and 43-bp truncated copies of IS*Aba14* located, respectively, 154 bp upstream and 844 bp downstream from the gene (Fig. 2; see also Table S1 in the supplemental material). In order to determine the right and left regions flanking this small fragment, the sequences upstream and downstream from

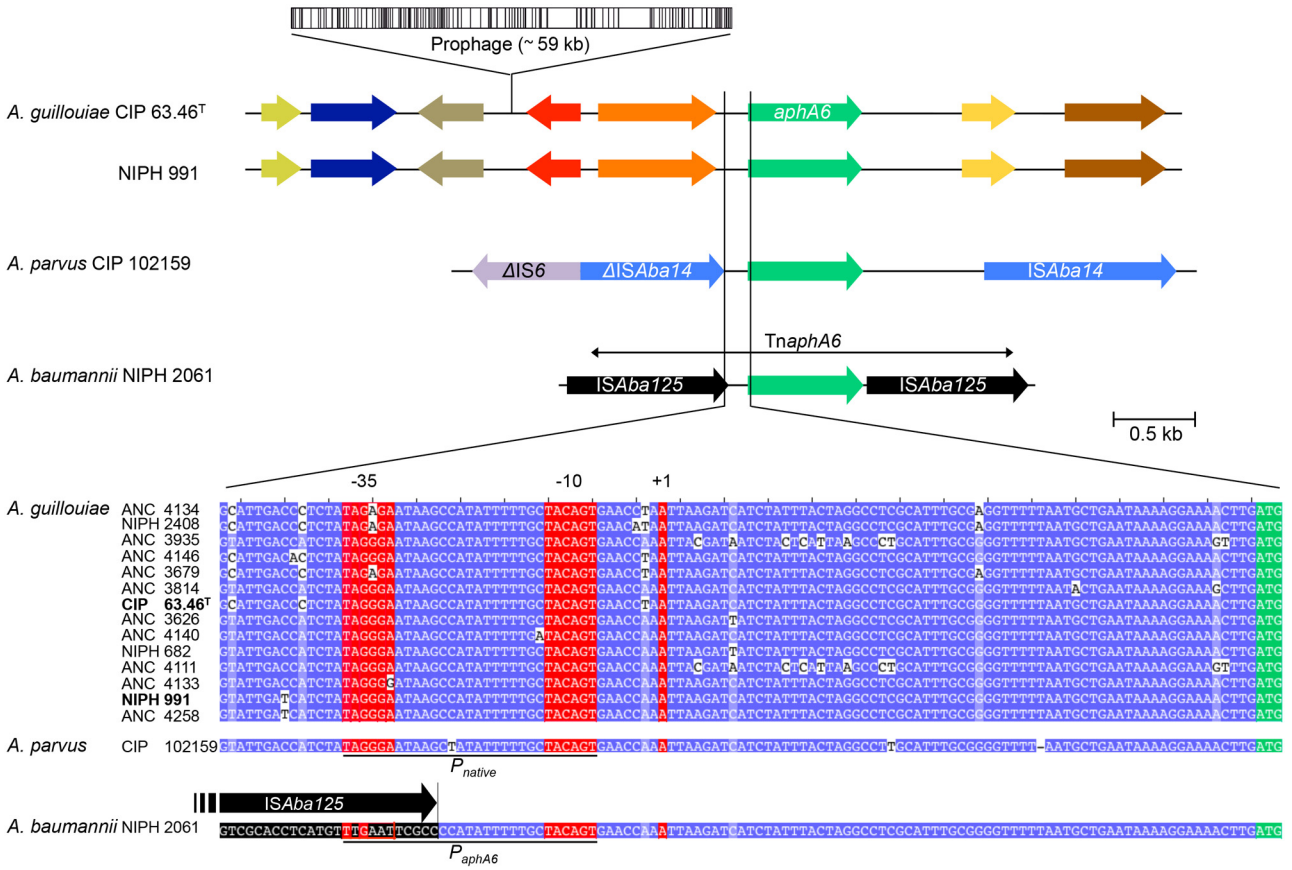

FIG 2 Genomic environment and promoters of *aphA6*. (Top) Arrows indicate open reading frames and sense of transcription. Open box, prophage; colored arrows, ORFs; green arrow, *aphA6*; purple arrow, IS*6*; blue arrow, IS*Aba14*; black arrow, IS*Aba125*; double-headed arrow, Tn*aphA6*. (Bottom) Sequence alignment of *aphA6* promoters from 14 *A. guillouiae*, *A. parvus* CIP 102159, and *A. baumannii* NIPH 2061 isolates. Identity between the sequences is indicated in blue. The P_{native} and P_{aphA6} hybrid promoters are underlined; the −35 and −10 sequences and the transcriptional start site (+1) as determined by 5′ RACE-PCR are in red boxes; the start codon of *aphA6* is in a green box. Boldface indicates the whole genome sequence of *A. guillouiae*. The promoter of *A. baumannii* NIPH 2061 was determined previously (16).

aphA6 were determined by using thermal asymmetric interlaced PCR (TAIL-PCR). The presence of the adjacent IS*Aba14* was confirmed, with the left copy, beginning at position 343, truncated, whereas the right copy was complete (1,282 bp) (see Fig. S2 in the supplemental material). Comparative analysis revealed 21 base changes between the two IS. Conventional PCR with primers 102159-Fi and 102159-Ri (see Table S2 and Fig. S2 in the supplemental material) complementary to sequences outside the ΔIS*Aba14-aphA6*-IS*Aba14* region confirmed the TAIL-PCR results by amplification of the expected 4,198-bp fragment but also of a 1,140-bp fragment (see Fig. S2). Sequence determination of the latter revealed ΔIS*Aba14*, the result of the fusion of the 5′ portion of the left copy with the 3′ portion of the right copy, generated by recombination at position 510 based on the substitutions between the two IS. In addition, a circular form containing *aphA6* and IS*Aba14* with the expected mutations of the two copies, which represented the excision product following the homologous recombination event, was also found by using inverse PCR carried out with primers 102159-R1 and 102159-F1 (see Table S2). PCR amplification with primers 102159-Fi and 102159-Ri (see Table S2 and Fig. S2) on genomic DNA from six *aphA6*-negative *A. parvus* strains confirmed the presence of a ΔIS*Aba14* target for integration of the gene. In summary, analysis of the genomic environment of *aphA6* in *A. parvus* allowed detection of the gene integrated in the chromosome and when excised, and also to identify the target IS element for integration.

Resistance conferred by Aph(3′)-VI. In contrast to other Aph(3′) proteins from Gram-negative bacteria, the type VI enzyme confers resistance, not only to kanamycin but also to amikacin (12). Throughout the whole-genome analysis, it was found that *A. baumannii* strains NIPH 290, NIPH 1669, NIPH 1734, and MRSN 3405 encode additional aminoglycoside-modifying enzymes, such as Ant(2″)-Ia and Aph(3′)-I, whose substrates include kanamycin. In addition, *A. baumannii* NIPH 290 and MRSN 3405 harbored five and two *aphA6* copies, respectively (see Table S1 in the supplemental material). *A. baumannii* NIPH 2061, which had a single copy of *aphA6* and no other aminoglycoside resistance gene, was thus selected for further studies. As expected, this strain had high MICs of kanamycin, 1,024 μg/ml, and amikacin, 128 μg/ml. In contrast, *A. guillouiae* CIP 63.46^T and NIPH 991 had low kanamycin MICs, of 16 and 1 μg/ml, respectively, and remained susceptible to amikacin, with MICs of 1 and 0.5 μg/ml, respectively (18, 19). The aminoglycoside MICs against *A. parvus* CIP 102159 could not be determined due to extremely slow growth of the strain. The susceptibilities to kanamycin and amikacin of the 29 additional *aphA6*-containing *A. guillouiae* strains were deter-

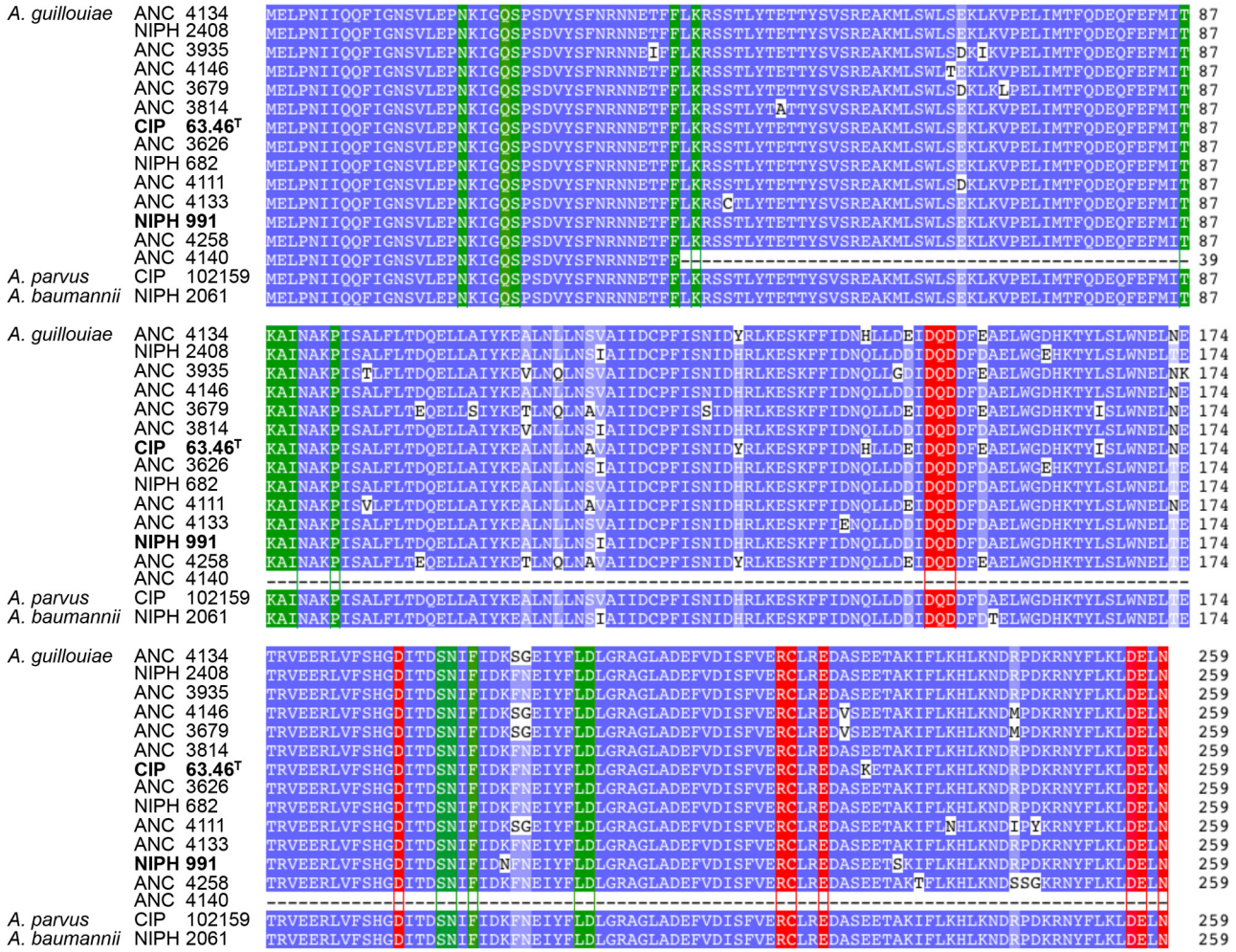

FIG 3 Comparison of Aph(3′)-VI sequences. Multiple alignment of the deduced sequences of Aph(3′)-VI from *A. guillouiae* and *A. parvus* CIP 102159 and strain NIPH 2061, representatives of the five identical *A. baumannii* genes. Amino acids are shaded according to percent identity; darker amino acids are more highly conserved. Red box, antibotic binding site; green box, ATP binding site (20).

mined (Table 2), and the strains did not produce other aminoglycoside-modifying enzymes that might affect amikacin activity (data not shown). The MICs of kanamycin against the strains ranged from 0.5 to >32 μg/ml, but all the strains remained susceptible (MICs ≤8 μg/ml) to amikacin (18, 19).

Two possibilities could account for the unexpected susceptibility of the *aphA6*-carrying *A. guillouiae* strains: (i) mutations affecting the enzyme activity and (ii) low levels of gene expression.

The sequences of the *aphA6* genes in the five *A. baumannii* strains were nearly identical (Table 1). The deduced sequences of Aph(3′)-VI from the two *A. guillouiae* strains and of *A. baumannii* NIPH 2061 were compared, together with the deduced sequence of *A. parvus* and of 12 additional *A. guillouiae* strains (Fig. 3). Despite various substitutions, we did not find any mutations in the residues that were part of the active site (20).

Resistance correlates with *aphA6* expression. The level of *aphA6* expression in the same 14 *A. guillouiae* isolates was determined by quantitative reverse transcriptase PCR (qRT-PCR), normalized to that of *rpoB*, and compared with the kanamycin and amikacin MICs (Fig. 4). The *A. baumannii* strains had *aphA6* expression levels 10^2- to 10^6-fold higher than those of the *A. guil-*

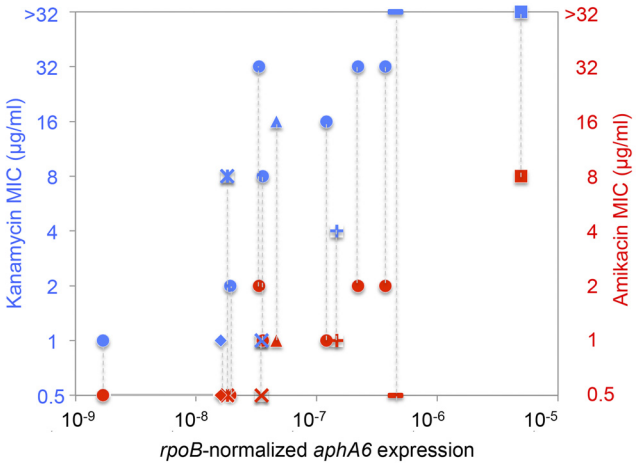

FIG 4 Aminoglycoside MICs and *aphA6* expression. The MICs of kanamycin (blue) and amikacin (red) against 14 *A. guillouiae* strains were determined by agar dilution, and *rpoB*-normalized *aphA6* expression was measured by qRT-PCR in duplicate in two independent experiments. ■, ANC 4134; ◆, NIPH 991; ▲, CIP 63.46T; —, NIPH 2408; X, ANC 4140; +, ANC 3679; *, ANC 4133; ●, remaining *A. guillouiae* strains.

TABLE 3 Susceptibility to aminoglycosides

Strain/plasmid[a]	MIC (μg/ml)[b]	
	Kanamycin	Amikacin
E. coli TOP10	2	1
E. coli TOP10/pAT747 (pUC18Ω*ori* pWH1266)	2	1
A. baumannii BM4587	2	2
A. baumannii BM4587/pAT747 (pUC18Ω*ori* pWH1266)	2	2
A. guillouiae CIP 63.46^T	16	1
E. coli TOP10/pAT747ΩP_{native}*aphA6 A. guillouiae* CIP 63.46^T	512	32
E. coli TOP10/pAT747ΩP_{aphA6}*aphA6 A. guillouiae* CIP 63.46^T	2,048	128
A. baumannii BM4587/pAT747ΩP_{native}*aphA6 A. guillouiae* CIP 63.46^T	1,024	128
A. baumannii BM4587/pAT747ΩP_{aphA6}*aphA6 A. guillouiae* CIP 63.46^T	4,096	1,024
A. guillouiae NIPH 991	1	0.5
E. coli TOP10/pAT747ΩP_{native}*aphA6 A. guillouiae* NIPH 991	32	4
E. coli TOP10/pAT747ΩP_{aphA6}*aphA6 A. guillouiae* NIPH 991	256	16
A. baumannii BM4587/pAT747ΩP_{native}*aphA6 A. guillouiae* NIPH 991	512	64
A. baumannii BM4587/pAT747ΩP_{aphA6}*aphA6 A. guillouiae* NIPH 991	2,048	256
A. parvus CIP 102159	NG[c]	NG
E. coli TOP10/pAT747ΩP_{native}*aphA6 A. parvus* CIP 102159	2,048	256
A. baumannii BM4587/pAT747ΩP_{native}*aphA6 A. parvus* CIP 102159	4,096	1,024
A. baumannii NIPH 2061	1,024	128
E. coli TOP10/pAT747ΩP_{aphA6}*aphA6 A. baumannii* NIPH 2061	2,048	128
A. baumannii BM4587/pAT747ΩP_{aphA6}*aphA6 A. baumannii* NIPH 2061	4,096	1,024

[a] MICs were determined by microdilution (18, 19).
[b] P_{native}, native promoter in the strain; P_{aphA6}, hybrid promoter generated by insertion of IS*Aba125* in *A. baumannii*
[c] NG, no growth.

louiae strains. There was good correlation between the aminoglycoside MICs and the *aphA6* expression levels in the *A. guillouiae* strains, and the kanamycin MICs were always higher than those of amikacin. *A. guillouiae* ANC 4134, NIPH 2408, and ANC 3679 had the same mutation, G→A, in the P_{native} −35 sequence (Fig. 2), very high MICs of kanamycin (Table 2), and their genes were the most highly expressed (Fig. 4). Interestingly, *A. guillouiae* ANC 4134, which had respective kanamycin and amikacin MICs of >32 and 8 μg/ml, expressed *aphA6* at a level intermediate between the levels for the *A. guillouiae* and *A. baumannii* strains. There was good agreement between kanamycin and amikacin MICs.

To compare the strength of the P_{native} and P_{aphA6} promoters, the genes from *A. guillouiae* CIP 63.46^T and NIPH 991 and that of *A. baumannii* NIPH 2061 (Table 3) were cloned under the control of the P_{native} promoter or that of P_{aphA6} in shuttle vector pAT747 (pUC18Ω*ori*pWH1266) into *Escherichia coli* TOP10 and transferred to aminoglycoside-susceptible *A. baumannii* BM4587.

There were no changes in aminoglycoside resistance of *E. coli* TOP10 or *A. baumannii* BM4587 after acquisition of pAT747 (Table 3). The genes from *A. guillouiae* were expressed and functional under the control of their respective P_{native} promoter in both *E. coli* and *A. baumannii*, where they conferred resistance not only to kanamycin but also to amikacin. However, they conferred much higher levels of resistance (4 to 8 times higher) when expressed from the hybrid P_{aphA6}. The MICs of kanamycin and amikacin were 4 to 16 times higher against *E. coli* and *A. baumannii* harboring the constructs with *aphA6* from CIP 63.46^T compared to those containing the gene from NIPH 991, reflecting the difference in levels of resistance of the respective *A. guillouiae* strains (Table 3). Since this difference was also observed in plasmids for which the genes were expressed from P_{aphA6}, it is likely that it was not due to the fact that the CIP 63.46^T gene was expressed at two-fold-higher levels (Fig. 4).

As expected, the MICs of kanamycin were always higher than those of amikacin. Amikacin is a derivative of kanamycin A that has an aminohydroxybutyric acid substitution at position 1 of the deoxystreptamine ring, which partially blocks modification at the 3′- and 2″-hydroxyl and 3-amino groups.

Unexpectedly, the MICs of both drugs were always higher against the *A. baumannii* than the *E. coli* hosts, despite the fact that the plasmid copy number, determined by qPCR, was much more elevated in *E. coli* (ca. 250 copies per genome equivalent) than in *A. baumannii* (ca. 20 copies). This confirmed the notion of the importance of the bacterial host background for expression of resistance, and it constitutes additional indirect evidence for the origin of *aphA6* in an *Acinetobacter* species.

The *aphA6* gene from *A. parvus* was also readily expressed in both new hosts, and more so in *A. baumannii* (Table 3). Heterologous expression of *aphA6* from *A. guillouiae* NIPH 991 and *A. parvus* CIP 102159 was as efficient as that from *A. baumannii* NIPH 2061, as judged by the levels of resistance achieved.

Dissemination of *aphA6*. Phylogenetic trees of *aphA6* containing *Acinetobacter* spp. were constructed by using the partial sequence (861 bp) of the *rpoB* (Fig. 1) or *aphA6* gene (see Fig. S3 in the supplemental material). The *rpoB* tree was congruent with the results of the previous analysis (Fig. 1), presenting three distinct clades of the respective species (see Fig. S3A). However, that of *aphA6* generated a single group (see Fig. S3B), which was consistent with the high degree of sequence similarity observed between the enzymes of the three species (Table 1). The marked differences in topologies of the trees strongly support horizontal transfer of the *aphA6* gene within *Acinetobacter* spp.

Together with Aac(6′), the Aph(3′) proteins are the most widely distributed aminoglycoside-modifying enzymes in human bacterial pathogens (10). We have comparatively analyzed the various types (isozyme forms) and subtypes (resistance profiles

conferred) of Aph(3′) enzymes found in Gram-positive and -negative bacteria (see Fig. S4 in the supplemental material). This allowed us to clearly distinguish the known types and subtypes and also to identify two new types, VIII in *A. rudis* and IX in *A. gerneri*, which are closely related to but distinct from type VI (our unpublished data). The Aph(3′)-VI proteins, one of the most widely spread types, were monophyletic with more than 92.7% sequence identity and were intertwined in various genera, confirming recent horizontal transfer of the corresponding genes, not only among *Acinetobacter* spp. but also to phylogenetically remote Gram-negative bacteria, such as *Enterobacteriaceae* and *P. aeruginosa* (see Fig. S4 in the supplemental material). Analysis of the sequences surrounding *aphA6* (see Fig. S5 in the supplemental material) indicated that the gene was generally carried by self-transferable plasmids and often flanked by an IS or remnants of an IS, most often IS*Aba125* and IS*Aba14*, and less frequently IS*Pa14*; the three genetic elements are commonly found in the genomes of *Acinetobacter* spp., in particular in that of *A. parvus*, where *aphA6* reversibly integrates in the chromosome and could represent an intermediate in transfer (Fig. 1).

DISCUSSION

The structural gene *aphA6* is of clinical importance in Gram-negative bacteria since it encodes an enzyme, Aph(3′)-VI, which modifies amikacin, the most potent aminoglycoside in therapy of infections due to *Acinetobacter* spp. The gene, which was first detected in *A. baumannii* (12), has disseminated to numerous members of the family *Enterobacteriaceae* and to *P. aeruginosa* (see Fig. S4 in the supplemental material) by a combination of plasmid transfer (3) and transposition (4). We suggested an origin in this genus for *aphA6*, based on the base composition of the gene and on its physical link with an insertion sequence common in *Acinetobacter* spp.

The gene was part of the chromosome of the two *A. guillouiae* isolates in this study (see Table S1 in the supplemental material) and is present in the vast majority of the additional strains of this species screened by PCR (Table 2), confirming that it is ancestral to *A. guillouiae*. This raises the question of the physiological role of Aph(3′)-VI insofar as the corresponding gene is poorly expressed (Table 3 and Fig. 4) and can be lost (Table 2). Since it was not found in the other *Acinetobacter* spp., with the exception of a single strain of *A. parvus* and of the *A. baumannii* strains where, as already mentioned, it is acquired and part of transposable elements (Fig. 2; see also Fig. S5 in the supplemental material) (13, 14), this confirms that *A. guillouiae* is a likely source for *aphA6*.

The reservoir species is one of the most prevailing *Acinetobacter* species in natural environments, as revealed in the current prospective study focused on cultivable *Acinetobacter* strains in soil and water ecosystems in the Czech Republic which are not directly associated with human activity (A. Nemec and L. Krizova, unpublished data).

The mechanism for intra- and intergeneric transfer of the amikacin resistance gene from *A. guillouiae* to other *Acinetobacter* spp., and to members of the family *Enterobacteriaceae* and *P. aeruginosa*, remains unknown. However, and importantly, *A. guillouiae* is occasionally found also in human clinical specimens, which makes direct horizontal acquisition from this species to human pathogens conceivable. During this process, concomitant expression of the gene must have occurred, since it confers high-level resistance in the new hosts (Table 3) (12). A possible two-step scenario would be mobilization and expression of *aphA6* by the formation of a composite transposon, the upstream copy of the IS element generating the potent hybrid promoter P_{aphA6} (Fig. 2), followed by lateral transfer by plasmid conjugation. IS*Aba125* suggests itself as a prominent candidate for this dual role since, in addition to providing a −35 promoter sequence (Fig. 2) (16), it is widely spread in *Acinetobacter* spp., including *A. guillouiae* (Fig. 1), and commonly associated with *aphA6* in the recipients (see Fig. S5 in the supplemental material) (14). Decryption of the silent *aac*(6′)-*Ij* gene intrinsic to *Acinetobacter* sp. 13 by IS*18* led to resistance to amikacin (21), and that of the *ampC* gene leading to cephalosporin resistance following insertion of IS*Aba125* has been reported in *A. baumannii* (22).

It has been proposed that the only transfer of a bla_{OXA} gene for a class D β-lactamase within the *Acinetobacter* genus was from genomic species 6 to *A. parvus* (15). Remarkably, recipient *A. parvus*, despite the fact it has one of the smallest genomes among *Acinetobacter* spp., contains the 14 key type IV pilus and competence-associated components (M. Touchon et al., submitted for publication), and transformation could thus be a mechanism for acquisition of foreign genetic information. The *aphA6* gene in *A. parvus* is part of the remnant of a composite transposon with two terminal direct copies of IS*Aba14* (Fig. 2) that are able to mediate excision and integration of the gene. This structure is compatible with integration of *aphA6* by homologous recombination between the two IS following transformation. Alternatively, *aphA6* could have been spread by transduction, since we found, upstream from the gene in *A. guillouiae* CIP 63.46$^{\mathrm{T}}$, the remnants of a large prophage; we recently showed that prophages are very common in *Acinetobacter* species (M. Touchon et al., submitted for publication). Whatever the actual mechanism of transfer, it must have been a recent direct event, since the promoters and regions upstream from *aphA6* are perfectly conserved in *A. guillouiae*, *A. parvus*, and *A. baumannii* (Fig. 2).

The recently described metallo-β-lactamase NDM-1 confers resistance to all β-lactams except aztreonam. It has been convincingly shown that $bla_{\mathrm{NDM\text{-}1}}$ is a chimeric gene resulting from a very recent in-frame fusion between the N-terminal portion of *aphA6* extending to P_{aphA6} and a β-lactamase gene that occurred in an *Acinetobacter* sp. (17). It has also been proposed that worldwide dissemination of $bla_{\mathrm{NDM\text{-}1}}$ is, at least in part, due to control of its expression by the strong hybrid promoter P_{aphA6}, which has broad-host-range activity (17). The $bla_{\mathrm{NDM\text{-}1}}$ and *aphA6* genes are often physically linked on plasmids in Gram-negative bacteria, and their dissemination could therefore be the result of coselection.

It has been proposed that certain antibiotic resistance genes in human bacterial pathogens originated from antibiotic-producing microorganisms, which have to protect themselves against suicide (23). However, there are a few examples of species reservoirs of resistance genes in which, like *aphA6* in *A. guillouiae*, the gene is not (or poorly) expressed, e.g., *Acinetobacter radioresistens* for $bla_{\mathrm{OXA\text{-}23}}$ (24), *Kluyvera* spp. for bla_{CTX} for CTX-M-type extended-spectrum β-lactamases (25), and *Shewanella algae* for quinolone resistance determinant *qnrA* (26). Based on the data presented here, it thus appears that such genes should be searched for, not only in resistant (27) but also in environmental susceptible nonpathogenic bacteria.

MATERIALS AND METHODS

Bacterial strains. The whole-genome sequences of 133 *Acinetobacter* strains, including 13 genomes that are reported in GenBank RefSeq (28), 118 strains from the collection of A. Nemec (designated NIPH or ANC) and of the Institut Pasteur (designated CIP), *A. baumannii* MRSN 3405 (29), and BM4587 (30) were studied (Fig. 1). The strains were selected to reflect the currently known breadth of the diversity of the genus *Acinetobacter* at the species level (15). In addition, 36 strains of *A. guillouiae*, chosen to represent different sources, including human and animal specimens as well as water and soil samples obtained from natural ecosystems, were screened for the presence of the *aphA6* gene (Table 2). Of these 36 strains, 15 were from the original study that described *A. guillouiae* (31), while 21 were selected from an as-yet-unpublished taxonomic study focused on the distribution of cultivable *Acinetobacter* strains in the natural ecosystems of the Czech Republic (Nemec and Krizova, unpublished). The 21 environmental strains were identified as *A. guillouiae* based on comprehensive phenotypic testing, *rpoB* gene comparative analysis, and whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry profiling (31, 32; Nemec and Krizova, unpublished). *E. coli* One Shot TOP10 (Invitrogen) and *A. baumannii* BM4587 were used as recipients for cloning the *aphA6* gene. Bacteria were grown according to their physiological requirements, at 30°C to 37°C, in brain heart infusion broth and agar (Difco Laboratories).

Antimicrobial susceptibility testing. MICs were determined by microdilution in Mueller-Hinton broth (Bio-Rad) or dilution in Mueller-Hinton II agar (BBL BD) (18, 19).

Sequence analysis. The *aphA* genes inferred from whole-genome sequences obtained in this work or retrieved from GenBank were compared with known sequences by using the BLAST tool of the national Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov) (33). Multiple-sequence alignments and the amino acid identity calculation of the deduced protein sequences were carried out by using the MUSCLE program (multiple sequence comparison by log-expectation; http://www.ebi.ac.uk/Tools/msa/muscle/). Comparative nucleotide sequence analysis of the RNA polymerase β-subunit (*rpoB*) gene was performed according to methods described by Nemec et al. (32). Similarity calculations and cluster analyses were carried out using Bionumerics 6.6 software with default parameters for the region corresponding to nucleotide positions 2915 to 3775 of the *rpoB* coding region of *A. baumannii* CIP 70.34^T. The promoter and coding regions of *aphA6* in 12 of the 36 additional *A. guillouiae* strains were amplified with the aphA6-up200 and aphA6-down80 primers (see Table S2 in the supplemental material), the products were sequenced with primers aphA6-up200, aphA6-down80, aphA6-R, and aphA6-F2, and the sequences were analyzed with the GCG sequence analysis software package (version 10.1; Genetics Computer Group, Madison, WI).

DNA manipulation and recombinant DNA techniques. Genomic DNA was extracted as described previously (15). DNA amplification was performed in a GeneAmp PCR 9700 system (PerkinElmer Cetus) with Phusion high-fidelity DNA polymerase (Thermo Scientific). The PCR products were purified with a QIAquick PCR purification kit (Qiagen). Digestion with restriction endonucleases (New England Biolabs), 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). Nucleotide sequencing was carried out with a CEQ 8000 DNA analysis system automatic sequencer (Beckman Instruments).

Identification of the *aphA6* gene. The list of putative homologs between pairs of genomes was determined with blastp based on the sequence of Aph(3′)-VI (34). The presence of *aphA6* in the additional *A. guillouiae* strains was determined by PCR using primers aph3-VIA1 and -VIA2 (see Table S2 in the supplemental material) (35). The reactions were performed in a final volume of 20 μl containing 10 μl of *Taq* PCR master mix (Qiagen), 0.2 μM of each primer, and 1.5 μl of a DNA suspension obtained by alkaline lysis. The amplification reactions were performed with the following parameters: 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C.

RNA isolation and qRT-PCR. Total RNA was extracted from exponentially growing bacterial cells (optical density at 600 nm, ≈0.8) by using TRIzol reagent (Invitrogen) (30). RNA samples were treated using the Turbo DNA-free kit (Applied Biosystems) to remove any genomic DNA carryover. Expression of the *aphA6* and *rpoB* genes was quantified by qRT-PCR using a LightCycler RNA amplification kit with SYBR green I (Roche Diagnostics) with the following cycle profile: 1 cycle at 95°C for 30 s, followed by 45 cycles at 95°C for 5 s, 56°C for 10 s, and 72°C for 20 s. The primers used are listed in Table S2 in the supplemental material. The transcriptome of *aphA6* was normalized to that of *rpoB*, and each experiment was performed in duplicate at least twice independently.

Construction of *A. baumannii-E. coli* shuttle vector pAT747. A 1,349-bp fragment containing the replication origin of plasmid pWH1266 (36) was amplified with primers ori-pWH-AflIII-F and ori-pWH-AflIII-R (see Table S2 in the supplemental material), digested by AflIII, and ligated to AflIII-linearized pUC18 to generate pAT747, which was transformed into *E. coli* TOP10 cells with selection on medium containing ampicillin at 100 μg/ml.

Cloning of *aphA6* genes. The 1,060-bp fragment that included 175 bp upstream from the −35 motif of the *aphA6* promoter was amplified from *A. baumannii* NIPH 2061 with primers ISaba125-BamHI-F and aphA6-XbaI-R (see Table S2 in the supplemental material). Similarly, 1,091-bp fragments encompassing 195 bp upstream from the −35 sequence of the promoter were amplified from *A. guillouiae* CIP 63.46^T and NIPH 991 by using aphA6-guillouiae-BamHI-F and aphA6-XbaI-R primers (see Table S2), and the BamHI-XbaI-digested products were ligated to BamHI-XbaI-linearized pAT747. The 1,081-bp fragment was amplified from *A. parvus* CIP 102159 by using aphA6-parvus-F and aphA6-XbaI-R primers (see Table S2) and digested by XbaI, and the fragment including 168 bp upstream from the −35 sequence of the promoter was ligated to XbaI-linearized pAT747. Plasmids pAT747ΩP_{aphA6}*aphA6* were constructed by successive PCR. First, the 295-bp P_{aphA6} fragment was amplified from *A. baumannii* NIPH 2061 with primers ISaba125-BamHI-F and aphA6-comp-R, and the 795-bp *aphA6* genes from *A. guillouiae* NIPH 991 and CIP 63.46^T were amplified with primers aphA6-comp-F and aphA6-XbaI-R. In the second step, the two products were linked in 1,076-bp fragments by overlapping PCR using primer pair ISaba125-BamHI and aphA6-XbaI-R to obtain P_{aphA6}*aphA6*, which was digested with BamHI and XbaI and cloned into pAT747. The plasmids were transformed into *E. coli* TOP10 with selection on medium containing ampicillin at 100 μg/ml and kanamycin at 25 μg/ml and electrotransformed into *A. baumannii* BM4587 cells with selection on medium containing ticarcillin at 30 μg/ml and kanamycin at 25 μg/ml. The orientations and sequences of all the inserts were verified by using forward and reverse universal primers.

Determination of the transcriptional start site. 5′-rapid amplification of cDNA ends PCR (5′ RACE-PCR) was carried out with the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instruction, using nested *aphA6* gene-specific primers aphA6-R and aphA6-R2 (see Table S2 in the supplemental material). The resulting amplified fragments were purified, cloned into pCR-Blunt (Invitrogen), and sequenced.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01972-14/-/DCSupplemental.

Text S1, DOCX file, 0.1 MB.
Figure S1, PDF file, 0.05 MB.
Figure S2, PDF file, 0.05 MB.
Figure S3, PDF file, 0.1 MB.
Figure S4, PDF file, 0.1 MB.
Figure S5, PDF file, 0.1 MB.
Table S1, DOCX file, 0.03 MB.
Table S2, DOCX file, 0.04 MB.

ACKNOWLEDGMENTS

We thank Martina Maixnerova for help with susceptibility testing.

E.-J.Y. was supported by an unrestricted grant from Reckitt-Benckiser, A.N. and L.K. were supported by grant 13-26693 S from the Czech Science Foundation, and M.T. was supported by European Research Council grant EVOMOBILOME 281605. This project was funded in part with federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no. HHSN272200900018C.

ADDENDUM IN PROOF

The article listed in the text as "submitted for publication" has since been accepted: M. Touchon, J. Cury, E. J. Yoon, L. Krizova, G. Cerqueira, C. Murphy, M. Feldgarden, J. Wortman, D. Clermont, T. Lambert, C. Grillot-Courvalin, A. Nemec, P. Courvalin, E. P. Rocha, Genome Biol. Evol., in press.

REFERENCES

1. **Dijkshoorn L, Nemec A, Seifert H.** 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat. Rev. Microbiol. **5:**939–951. http://dx.doi.org/10.1038/nrmicro1789.
2. **Turton JF, Shah J, Ozongwu C, Pike R.** 2010. Incidence of *Acinetobacter* species other than *A. baumannii* among clinical isolates of *Acinetobacter*: evidence for emerging species. J. Clin. Microbiol. **48:**1445–1449. http://dx.doi.org/10.1128/JCM.02467-09.
3. **Goldstein FW, Labigne-Roussel A, Gerbaud G, Carlier C, Collatz E, Courvalin P.** 1983. Transferable plasmid-mediated antibiotic resistance in *Acinetobacter*. Plasmid **10:**138–147. http://dx.doi.org/10.1016/0147-619X(83)90066-5.
4. **Devaud M, Kayser FH, Bächi B.** 1982. Transposon-mediated multiple antibiotic resistance in *Acinetobacter* strains. Antimicrob. Agents Chemother. **22:**323–329. http://dx.doi.org/10.1128/AAC.22.2.323.
5. **Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P, Weissenbach J, Raoult D, Claverie JM.** 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. PLoS Genet. **2:**e7. http://dx.doi.org/10.1371/journal.pgen.0020007.
6. **Adams MD, Chan ER, Molyneaux ND, Bonomo RA.** 2010. Genome-wide analysis of divergence of antibiotic resistance determinants in closely related isolates of *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **54:**3569–3577. http://dx.doi.org/10.1128/AAC.00057-10.
7. **Magnet S, Courvalin P, Lambert T.** 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. Antimicrob. Agents Chemother. **45:**3375–3380. http://dx.doi.org/10.1128/AAC.45.12.3375-3380.2001.
8. **Galimand M, Courvalin P, Lambert T.** 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob. Agents Chemother. **47:**2565–2571. http://dx.doi.org/10.1128/AAC.47.8.2565-2571.2003.
9. **Magnet S, Smith TA, Zheng R, Nordmann P, Blanchard JS.** 2003. Aminoglycoside resistance resulting from tight drug binding to an altered aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. **47:**1577–1583. http://dx.doi.org/10.1128/AAC.47.5.1577-1583.2003.
10. **Lambert T.** 2010. Aminoglycosides and gram-negative bacteria, p 2251577–242. *In* Courvalin P, Leclercq R, Rice LB (ed), Antibiogram. ASM Press, Washington, DC.
11. **Nemec A, Dolzani L, Brisse S, van den Broek P, Dijkshoorn L.** 2004. Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among 2 strains of pan-European *Acinetobacter baumannii* clones. J. Med. Microbiol. **53:**1233–1240. http://dx.doi.org/10.1099/jmm.0.45716-0.
12. **Lambert T, Gerbaud G, Courvalin P.** 1988. Transferable amikacin resistance in *Acinetobacter* spp. due to a new type of 3′-aminoglycoside phosphotransferase. Antimicrob. Agents Chemother. **32:**15–19. http://dx.doi.org/10.1128/AAC.32.1.15.
13. **Lambert T, Gerbaud G, Courvalin P.** 1994. Characterization of transposon Tn*1528*, which confers amikacin resistance by synthesis of aminoglycoside 3′-O-phosphotransferase type VI. Antimicrob. Agents Chemother. **38:**702–706. http://dx.doi.org/10.1128/AAC.38.4.702.
14. **Nigro SJ, Post V, Hall RM.** 2011. Aminoglycoside resistance in multiply antibiotic-resistant *Acinetobacter baumannii* belonging to global clone 2 from Australian hospitals. J. Antimicrob. Chemother. **66:**1504–1509. http://dx.doi.org/10.1093/jac/dkr163.
15. **Périchon B, Goussard S, Walewski V, Krizova L, Cerqueira G, Murphy C, Feldgarden M, Wortman J, Clermont D, Nemec A, Courvalin P.** 2014. Identification of 50 class D beta-lactamases and 65 *Acinetobacter*-derived cephalosporinases in *Acinetobacter* spp. Antimicrob. Agents Chemother. **58:**936–949. http://dx.doi.org/10.1128/AAC.01261-13.
16. **Dortet L, Nordmann P, Poirel L.** 2012. Association of the emerging carbapenemase NDM-1 with a bleomycin resistance protein in *Enterobacteriaceae* and *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **56:**1693–1697. http://dx.doi.org/10.1128/AAC.05583-11.
17. **Toleman MA, Spencer J, Jones L, Walsh TR.** 2012. bla_{NDM-1} is a chimera likely constructed in *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **56:**2773–2776. http://dx.doi.org/10.1128/AAC.06297-11.
18. **Clinical and Laboratory Standards Institute.** 2012. Performance standards for antimicrobial susceptibility testing, vol -M100–MS22. Clinical and Laboratory Standards Institute, Wayne, PA.
19. **European Committee on Antimicrobial Susceptibility Testing.** 2014. Breakpoint tables for interpretation of MICs and zone diameters, version 4.0. http://eucast.org/clinical_breakpoints/. Accessed 18 January 2014.
20. **Fong DH, Berghuis AM.** 2002. Substrate promiscuity of an aminoglycoside antibiotic resistance enzyme via target mimicry. EMBO J. **21:**2323–2331. http://dx.doi.org/10.1093/emboj/21.10.2323.
21. **Rudant E, Courvalin P, Lambert T.** 1998. Characterization of IS*18*, an element capable of activating the silent *aac*(*6*′)-*Ij* gene of *Acinetobacter* sp. 13 strain BM2716 by transposition. Antimicrob. Agents Chemother. **42:**2759–2761.
22. **Hamidian M, Hancock DP, Hall RM.** 2013. Horizontal transfer of an IS*Aba125*-activated *ampC* gene between *Acinetobacter baumannii* strains leading to cephalosporin resistance. J. Antimicrob. Chemother. **68:**244–245. http://dx.doi.org/10.1093/jac/dks345.
23. **Courvalin P, Weisblum B, Davies J.** 1977. Aminoglycoside-modifying enzyme of an antibiotic-producing bacterium acts as a determinant of antibiotic resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. **74:**999–1003. http://dx.doi.org/10.1073/pnas.74.3.999.
24. **Poirel L, Figueiredo S, Cattoir V, Carattoli A, Nordmann P.** 2008. *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. Antimicrob. Agents Chemother. **52:**1252–1256. http://dx.doi.org/10.1128/AAC.01304-07.
25. **Humeniuk C, Arlet G, Gautier V, Grimont P, Labia R, Philippon A.** 2002. Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob. Agents Chemother. **46:**3045–3049. http://dx.doi.org/10.1128/AAC.46.9.3045-3049.2002.
26. **Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A, Nordmann P.** 2005. Origin of plasmid-mediated quinolone resistance determinant QnrA. Antimicrob. Agents Chemother. **49:**3523–3525. http://dx.doi.org/10.1128/AAC.49.8.3523-3525.2005.
27. **Spanogiannopoulos P, Waglechner N, Koteva K, Wright GD.** 2014. A rifamycin inactivating phosphotransferase family shared by environmental and pathogenic bacteria. Proc. Natl. Acad. Sci. U. S. A. **111:**7102–7107. http://dx.doi.org/10.1073/pnas.1402358111.
28. **Pruitt KD, Tatusova T, Maglott DR.** 2007. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res. **35:**D61–D65. http://dx.doi.org/10.1093/nar/gkl842.
29. **Lesho E, Yoon EJ, McGann P, Snesrud E, Kwak Y, Milillo M, Onmus-Leone F, Preston L, St Clair K, Nikolich M, Viscount H, Wortmann G, Zapor M, Grillot-Courvalin C, Courvalin P, Clifford R, Waterman PE.** 2013. Emergence of colistin-resistance in extremely drug-resistant *Acinetobacter baumannii* containing a novel *pmrCAB* operon during colistin therapy of wound infections. J. Infect. Dis. **208:**1142–1151. http://dx.doi.org/10.1093/infdis/jit293.
30. **Coyne S, Guigon G, Courvalin P, Périchon B.** 2010. Screening and quantification of the expression of antibiotic resistance genes in *Acinetobacter baumannii* with a microarray. Antimicrob. Agents Chemother. **54:**333–340. http://dx.doi.org/10.1128/AAC.01037-09.
31. **Nemec A, Musílek M, Sedo O, De Baere T, Maixnerová M, van der Reijden TJ, Zdráhal Z, Vaneechoutte M, Dijkshoorn L.** 2010. *Acinetobacter bereziniae* sp. nov. and *Acinetobacter guillouiae* sp. nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively. Int. J. Syst. Evol. Microbiol. **60:**896–903. http://dx.doi.org/10.1099/ijs.0.013656-0.
32. **Nemec A, Krizova L, Maixnerova M, van der Reijden TJ, Deschaght P, Passet V, Vaneechoutte M, Brisse S, Dijkshoorn L.** 2011. Genotypic and

phenotypic characterization of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter* genomic species 13TU). Res. Microbiol. **162:**393–404. http://dx.doi.org/10.1016/j.resmic.2011.02.006.

33. **Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and psi-blast: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402. http://dx.doi.org/10.1093/nar/25.17.3389.
34. **Martin P, Jullien E, Courvalin P.** 1988. Nucleotide sequence of *Acinetobacter baumannii aphA-6* gene: evolutionary and functional implications of sequence homologies with nucleotide-binding proteins, kinases and other aminoglycoside-modifying enzymes. Mol. Microbiol. **2:**615–625. http://dx.doi.org/10.1111/j.1365-2958.1988.tb00070.x.
35. **Vila J, Ruiz J, Navia M, Becerril B, Garcia I, Perea S, Lopez-Hernandez I, Alamo I, Ballester F, Planes AM, Martinez-Beltran J, de Anta TJ.** 1999. Spread of amikacin resistance in *Acinetobacter baumannii* strains isolated in Spain due to an epidemic strain. J. Clin. Microbiol. **37:**758–761.
36. **Hunger M, Schmucker R, Kishan V, Hillen W.** 1990. Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. Gene **87:**45–51. http://dx.doi.org/10.1016/0378-1119(90)90494-C.