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Genetic Variability of AdeRS Two-Component System Associated with Tigecycline Resistance in XDR-*Acinetobacter baumannii* Isolates

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Abstract The emergence of tigecycline resistance has increased in the last years. Although tigecycline-resistant *Acinetobacter baumannii* isolates were described all over the world, few reports regarding the molecular basis of this resistance are available. It has been recognized that the overexpression of AdeABC efflux pump is related to the tigecycline-resistant phenotype. In 37 clinical *A. baumannii* isolates we first determined the tigecycline-resistant phenotype and then, within a selected group, we analyzed the sequence of the *adeRS* operon, which is involved in the expression of the AdeABC efflux pump. Nucleotide sequence analysis of *adeR* and *adeS* showed the presence of 5 and 16 alleles, respectively. These results expose a high genetic variability in both genes, the *adeS* gene being more susceptible to genetic variation. The presence of 2 AdeR and 2 AdeS new variants were reported. Two of the new AdeRS variants were present in the intermediate and the resistant tigecycline *A. baumannii* isolates, suggesting a putative role in the development of the observed phenotype. More studies need to be addressed to determine the role of the genetic variability observed in the *adeRS* operon.

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Introduction

In the last years, infections caused by XDR-*Acinetobacter baumannii* isolates were documented all over the world [1]. High levels of morbidity and mortality are associated with this important nosocomial pathogen [7]. The dispersion of International Clonal Lineages—such as ICL1, ICL2, ICL3, and CC25—with high level resistance as a relevant characteristic is involved in the success of this species as an important nosocomial pathogen [9–23]. In some cases colistin and tigecycline are the only drugs available to treat *A. baumannii*'s infections [1, 6–17].

Since there is an extensive use of tigecycline to combat XDR-*A. baumannii*'s infections in clinical settings, a rapid development of tigecycline resistance was observed [2]. Few reports regarding the molecular mechanisms of tigecycline resistance are available [3]. The development of this resistance was associated with the overexpression of different efflux pumps (AdeABC, AdeIJK, AdeFGH, AbeM, and AdeDE) and the presence of *tetX* gene [4, 17].

A two-component system containing a sensor kinase and a response regulator are responsible for modulating the expression of efflux pumps [3]. In the particular case of AdeABC efflux pump, it is tightly regulated by the *adeRS* operon where AdeS is the sensor and AdeR is the response regulator [3]. Nucleotide and amino acid variations, as well as the invasion of this operon by IS*Aba1*, have been related to the overexpression of the AdeABC efflux pump [10–19].

The aim of the present work was to investigate the tigecycline-resistant phenotype, the molecular epidemiology, and the sequence variation of the *adeRS* operon in our *A. baumannii* isolates.

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Materials and Methods

Thirty-seven XDR-*A. baumannii* isolates were studied. The isolates were recovered in the latest years (2011–2013) (Table 1) from different hospitals ($n = 6$) in Buenos Aires Argentina. All the isolates were categorized as extensively drug resistant (XDR) according to the recent definitions suggested by Magiorakos et al. [15].

To determine the tigecycline-resistant phenotype of the *A. baumannii* isolates, disk diffusion method and minimal inhibitory concentrations (MICs) were performed. The MIC was determined by agar dilution method using a standardized 0.5 McFarland standard inoculum on fresh Mueller–Hinton agar (Difco™) plates. The breakpoint criteria used to determine the tigecycline phenotype was based on the United States Food and Drug Administration breakpoint criteria used for *Enterobacteriaceae* considering susceptibility </=2 mg/L, intermediate at 4 mg/L, and resistance at >/=8 mg/L.

Total DNA was extracted and used to perform PCR amplification reactions according to manufacturer's instructions (Promega, Madison, WI, USA). The pan-PCR assay was used to define the relatedness among the studied *A. baumannii* isolates [22]. This assay was carried out as described by Yang et al. using the 6 designed pairs of primers that amplify a group of genes whose variable presence enables identification of the studied isolates [22]. PCR reactions to amplify *adeS, adeR, adeA, adeB, tet*(X), and IS*Aba1* were carried out using specific primers (Table 2). For the *adeS* gene amplification, 3 different pairs of primers were designed in order to confirm the presence of the gene in all isolates since some isolates gave negative results with the first pair of primers used (AdeSF and AdeSR) (Tabla 2).

All PCR amplification products were sequenced on both DNA strands using an ABIPrism 3100 BioAnalyzer and Taq FS Terminator Chemistry (Taq FS, Perkin-Elmer) and sequence analysis was performed with the Sequencher 4.7 software (Gene Codes Corp.), BLAST (version 2.0) software (http://www.ncbi.nlm.nih.gov/BLAST/), clustal X (version 2.1), Mega5, and the PVS tool (http://imed.med.ucm.es/PVS/).

Results and Discussion

Tigecycline Susceptibility Determination and Molecular Epidemiology of *A. baumannii* Isolates

As mentioned before, all *A. baumannii* isolates included in the present study were categorized as XDR [15].

To determine tigecycline susceptibility two different methods were used. First, the disk diffusion method—which is the method used in most clinical laboratories in Argentina. We used the criteria recommended by the US FDA for *Enterobacteriaceae* to define susceptibility, showing that 49 % of our *A. baumannii* isolates were resistant to tigecycline. Secondly, knowing the discrepancies observed for tigecycline susceptibility with this method, we proceeded to perform the MIC by the agar dilution method (Difco™ plates) and obtained 19 % (7/37) resistance among the studied isolates. Moreover, ten isolates (27 %) were categorized as intermediate with the last methodology. Our results reinforced the previously published reports on tigecycline susceptibility for *A. baumannii* isolates in which remarkable differences on tigecycline resistance levels have been documented depending on the method used [13, 14]. Here we clearly showed that the results for the diffusion method and agar dilution (Difco™ agar plates) are not equivalent. We consider that the disk diffusion method could be used as a screening method, as it shows no very major errors, and the resistant profile must be confirmed by another method.

The pan-PCR assay was used to determine the relationship of all the *A. baumannii* isolates (susceptible, intermediate, and resistant to tigecycline). Using this assay we observed the presence of 4 different amplification patterns, where the prevalent pattern (53 %) is the one that corresponds to Clonal Complex (CC) $CC109^{B}/CC1^{P}$. Remarkably, in 12 isolates (35 %) we observed the pan-PCR amplification pattern that corresponds to the $CC110^{B}/CC25^{P}$ pattern. Furthermore, only one isolate belonged to the $CC113^{B}/CC79^{P}$ and 3 isolates exposed a new pan-PCR pattern, designated as P133 that corresponded to a new singleton (Vilacoba E. unpublished 2014). In this study we observed an increase in the dispersion of $CC109^{B}/CC1^{P}$ and $CC110^{B}/CC25^{P}$, which have been well distributed in other countries [23] displacing the $CC113^{B}/CC79^{P}$ that had been prevalent in our country in previous years [18]. These results exposed a shift to clonal level, thus indicating a new regional epidemiology.

Detection and Molecular Variability of *adeR* and *adeS* Genes

Positive results for the amplification of the *adeRS* operon were obtained in 31 (84 %) of the *A. baumannii* isolates included in this study. In one isolate (Ab378) we observed the absence of the *adeR* gene and in three isolates (Ab336, Ab414, and Ab11948) the *adeS* gene was absent. Moreover, two isolates (Ab38371, Ab41384) were negative for the complete *adeRS* operon.

Nucleotide sequence analysis of the complete sequence of 28 *adeR* amplicons revealed the presence of 5 alleles, harboring from 0 to 14 synonymous mutations with respect

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Table 1 Isolates analyzed in this study

Strain	Year	Hospital	Pan-PCR pattern	Tigecycline phenotype[a]	
				Disk difussion (mm)	MIC (µg/ml)
AbM13205	2011	H1	CC110[B]/CC25[P]	20 (S)	<0.5 (S)
AbM13338	2011	H1	CC109[B]/CC1[P]	14 (R)	4 (I)
AbM13484	2011	H1	CC109[B]/CC1[P]	14 (R)	4 (I)
AbF14393	2011	H1	CC109[B]/CC1[P]	14 (R)	4 (I)
AbM15111	2011	H1	CC109[B]/CC1[P]	17 (I)	2 (S)
Ab47	2011	H1	CC109[B]/CC1[P]	14 (R)	2 (S)
AbF15424	2011	H1	CC109[B]/CC1[P]	13 (R)	4 (I)
AbM15975	2011	H1	CC109[B]/CC1[P]	13 (R)	4 (I)
AbM15799	2011	H1	CC109[B]/CC1[P]	13 (R)	>8 (R)
AbF15897	2011	H1	CC109[B]/CC1[P]	15 (I)	8 (R)
Ab42	2011	H1	CC109[B]/CC1[P]	15 (I)	4 (I)
Ab53	2011	H1	CC109[B]/CC1[P]	14 (R)	2 (S)
Ab103	2011	H1	CC109[B]/CC1[P]	12 (R)	4 (I)
Ab112	2011	H1	CC109[B]/CC1[P]	15 (I)	4 (I)
Ab120	2011	H1	CC109[B]/CC1[P]	13 (R)	2 (S)
Ab122	2010	H2	CC109[B]/CC1[P]	13 (R)	4 (I)
Ab132	2011	H2	CC110[B]/CC25[P]	15 (I)	2 (S)
Ab164	2011	H2	CC110[B]/CC25[P]	10 (R)	8 (R)
Ab64	2011	H2	CC109[B]/CC1[P]	17 (I)	2 (S)
Ab336	2011	H2	Singleton	19 (S)	1 (S)
Ab376	2011	H2	Singleton	18 (I)	2 (S)
Ab378	2011	H2	CC110[B]/CC25[P]	16 (I)	2 (S)
Ab389	2011	H2	CC109[B]/CC1[P]	19 (S)	2 (S)
Ab414	2011	H2	Singleton	18 (I)	2 (S)
Ab1049158	2013	H3	Singleton	19 (S)	<0.5 (S)
Ab1049648	2013	H3	CC110[B]/CC25[P]	22 (S)	2 (S)
Ab38471	2013	H4	CC110[B]/CC25[P]	20 (S)	1 (S)
Ab41384	2013	H4	CC113[B]/CC79[P]	22 (S)	<0.5 (S)
Ab40977	2013	H4	CC110[B]/CC25[P]	19 (S)	1 (S)
Ab109	2012	H5	CC109[B]/CC1[P]	13 (R)	8 (R)
Ab166	2012	H5	CC110[B]/CC25[P]	26 (S)	<0.5 (S)
AbM2	2013	H5	CC109[B]/CC1[P]	13 (R)	2 (S)
AbM16	2013	H5	CC110[B]/CC25[P]	26 (S)	<0.5 (S)
Ab13946	2013	NC1	CC109[B]/CC1[P]	14 (R)	4 (I)
Ab11917	2013	NC1	CC110[B]/CC25[P]	10 (R)	>8 (R)
Ab11948	2013	NC1	CC110[B]/CC25[P]	13 (R)	8 (R)
Ab13103	2013	CNR1	CC110[B]/CC25[P]	13 (R)	8 (R)

H hospital, *CN* National Center, *R* resistance, *I* intermediate, *S* susceptible, *MIC* minimum inhibitory concentration

* The breakpoint criteria used to determine the tigecycline phenotype was based on the United States Food and Drug Administration breakpoint criteria used for *Enterobacteriaceae*

to the closest neighbor (Table 3). We observed the presence of 9–21 nucleotide changes when we compared our sequences with the one present in the *A. baumannii* ATCC 17978 (AN YP_001084783). As in a previous work, we defined as variant when nucleotide differences are related to an amino acid change, and as alleles when nucleotide differences are synonymous or non-synonymous mutations [20]. The analysis of the 28 AdeR amino acidic sequences obtained exposed the presence of 5 different variants, 2 of them being the new AdeR variants (Fig. 1a). The other 3 variants are identical to AdeR sequences deposited in the GenBank DataBase (YP_001713700, WP_000459551,

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Table 2 Primers used in the PCR amplifications

Target	Oligonucleotide	Sequence (5′– 3′)	References
adeR	AdeRF	GTGGAGTAAGTGTGGAGAAATACG	This work
adeR	AdeRR	GAAATGAGCTTAAACTAATCCAGC	This work
adeS	AdeSF	GCGATGCACTAGAGCGAACCGTAG	This work
adeS	AdeSR	CAACAGGAAAATGCCACAAAATGGCGAG	This work
adeSnew	AdeSnewF	AGAATATTTTATGAAAGTAAGTTAG	This work
adeSnew	AdeSnewR	AGCGAATTACCCTATTTCTTCATGACC	This work
adeSint	AdeSintF	GCTGGATTAGTTTAAGCTCATTTC	This work
adeSint	AdeSintR	CTAGCTTAGCTTGATCCAAACGATC	This work
IS*Aba1*	ISAba1F	GTTATATCTTATCTTAAACA	Merkier 2008, Thesis
IS*Aba1*	ISAba1R	CCGATAAACTCACTGTCTGCGA	Merkier 2008, Thesis
tet(X)	TetXF	GAGACAACGACCGAGAGGCAAG	This work
tet(X)	TetXR	CGGCATCAAATGTGCGGCATC	This work
adeA	AdeAF	CAAGGTAGTGA AGTTAGAGCAG	This work
adeA	AdeAR	GATAGGACGAATTTCCGCTGTC	This work
adeB	AdeBF	CGTCAGCAAGGTTTACAGGTTG	This work
adeB	AdeBR	GAAGCCCTTCTTCTGGTGATG	This work

WP_000459543). The complete sequence of the new variants has been deposited in GenBank under accession numbers KJ710511 and KJ710512 for the Ab42 and Ab15111 isolates, respectively.

The AdeR histogram carried out with all the AdeR sequences available in the Genbank, to show the amino acid variations, was measured by Shannon entropy and exposed a conservation of amino acids along the sequence (Fig. 1b). However, in a deep analysis three highly conversed regions were identified in the amino acid sequence (Fig. 1b). We have designated these regions as highly conserved region 1 (HCR1), highly conserved region 2 (HCR2), and highly conserved region 3 (HCR3) (Fig. 1b).

For *adeS* amplification, 3 different pairs of primers and 16 good-quality partial sequences were analyzed. The analysis at nucleotide level of 16 *adeS* partial sequences revealed the presence of 16 alleles, showing a high level of nucleotide variability in comparison with *adeR* (5 alleles).

The analysis at protein level of the AdeS partial sequence (247 aa) exposed the presence of 3 different variants, 2 of them being the new AdeS variants (Fig. 2a) and the other one identical to sequence present in the AYE strain (accession number NC_010410). The complete sequence of the new variants has been deposited in GenBank under accession number KJ710514 and KJ710515 for the Ab109 and Ab120 isolates, respectively.

The AdeS histogram carried out with all the AdeS sequences available in the Genbank to show the amino acid variations among the AdeS partial sequences obtained in the present study, exposed a consistent conservation of amino acids along all the sequences (Fig. 2b).

Here we identified the alleles of the *adeR* and *adeS* genes found in our *A. baumannii* population and also a great variability within the nucleotide sequences of these genes is exposed. In an intermediate (Ab15111) and in a resistant (Ab42) tigecycline isolates, two novel variants of

Table 3 Features of the *adeR* alleles found in the *Acinetobacter baumannii* isolates

Isolate	Sequence comparison subject sequence (AN) nucleotide identity/amino acid identity (%)[a]	Total mutation/mutation synonymous/non-synonymous with respect to closest neighbor	Total mutation/mutation synonymous/non-synonymous with respect to operon AdeRS of *A. baumannii* ATCC17978
414	CP003967 99/99 %	9/7/2	23/22/1
42	CU459141 99/99 %	2/0/1 (+1 insertion)	13/11/1 (+1 insertion)
15424	CU459141 99/100 %	1/1/0	12/12/0
40799	CU459141 98/99 %	16/14/2	19/17/2
103	CU459141 100/100 %	0/0/0	10/10/0

* AN: GenBank accession number

[a] The percentage of identity in each allelic variant was defined against the most closed sequence in every case

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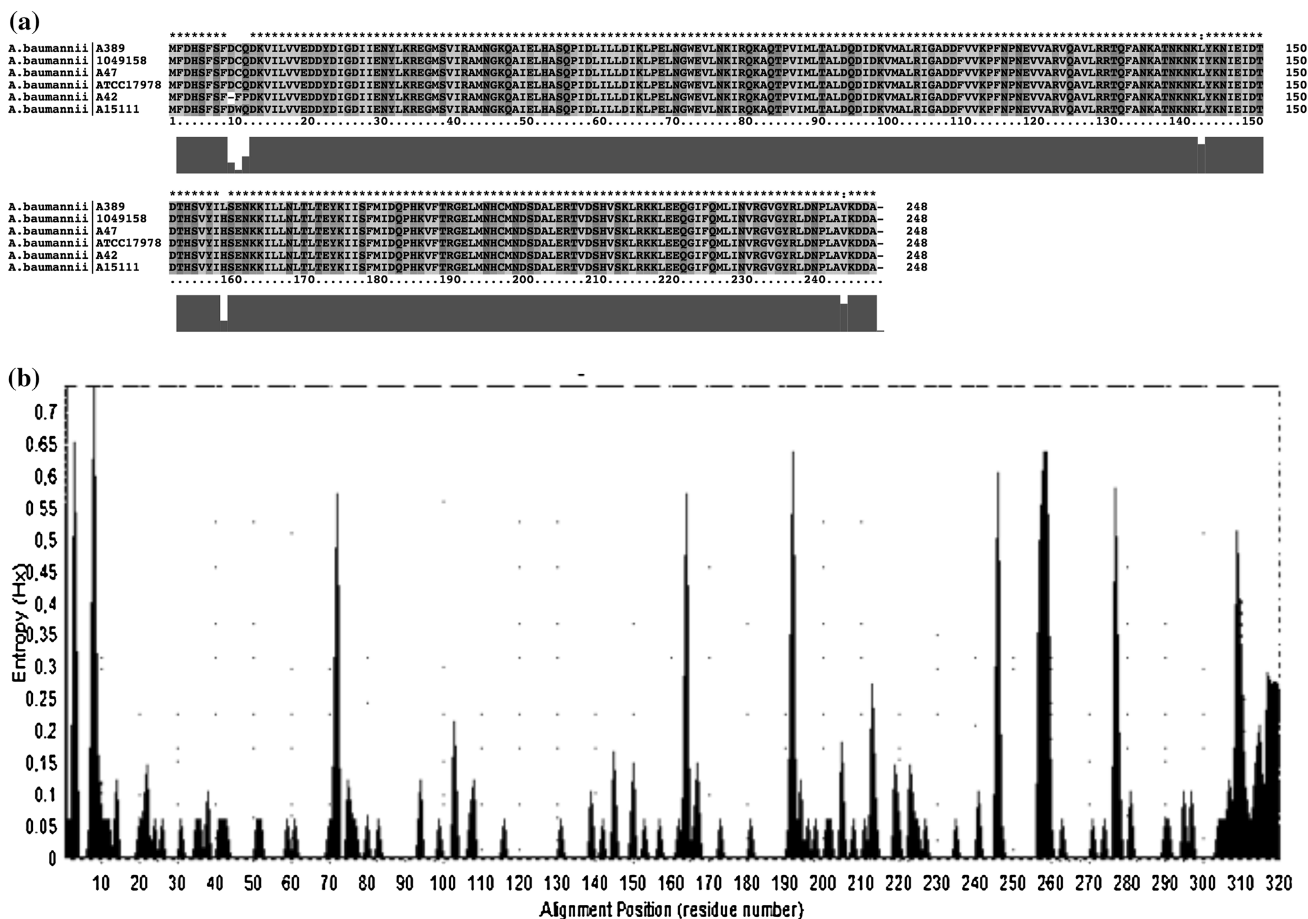

Fig. 1 **a** Amino acid sequence comparison of the AdeR sequence described in this study. The comparison was performed using the AdeR sequence of the *A. baumannii* ATCC 17978 strain as reference. The *stars* show identity among the amino acid sequences. **b** Histogram of AdeR sequence variability. The height of each *bar* indicates the degree of amino acid sequence variation from the consensus amino acid residue at that location, measured by the Shannon entropy for all AdeR proteins in the 28 *A. baumannii* clinical isolates analyzed in this study. The spans of highly conserved (HCR1, CHR2, and HCR3) *regions* are shown

AdeR were identified. Also, two novel variants of AdeS were found in the isolates Ab120 and Ab109 that are intermediate and resistant to tigecycline, respectively. The role of the described amino acid changes in the AdeR and AdeS sequences need to be determined. Here we described the genetic variability found in these genes as well as the alleles that are present in our population.

Taking into account the CCs present in this study and the *adeS* and *adeR* allele distribution, we did not observe a correlation between a particular CC and an *adeS* allele or an *adeR* allele. However, comparing the present results with the results obtained in a contemporary study carried out by our group with minocycline-resistant isolates, we observed that most of the tigecycline-resistant or tigecycline-intermediate isolates belonged to the CC109^{B}/CC1^{P}, while most of the minocycline-resistant or minocycline-intermediate isolates belonged to a new-ST or to CC110^{B}/CC25^{P} (Vilacoba E. unpublished 2014). The identification of the CCs with tigecycline resistance and/or minocycline resistance circulating in our hospitals gain insights in the epidemiology of the XDR isolates from our region.

Future studies will be done to address if there is any relation between the AdeRS variants described here and the development and/or contribution of tigecycline resistance. Since most of *A. baumannii* isolates recovered these days exhibit an extensively drug resistant (XDR) phenotype, tigecycline is a treatment option in some cases [5, 21]. Addressing the mechanisms that lead to tigecycline resistance will contribute to the knowledge of how this important pathogen could evolve to XDR and to pan-drug resistance.

Nucleotide Sequence Accession Number

The sequence of the novel variants of AdeS and AdeR sequences has been deposited in the GenBank under the accession numbers KJ710511 (Ab42), KJ710512 (Ab15111), KJ710514 (Ab109), and KJ710515 (Ab120).

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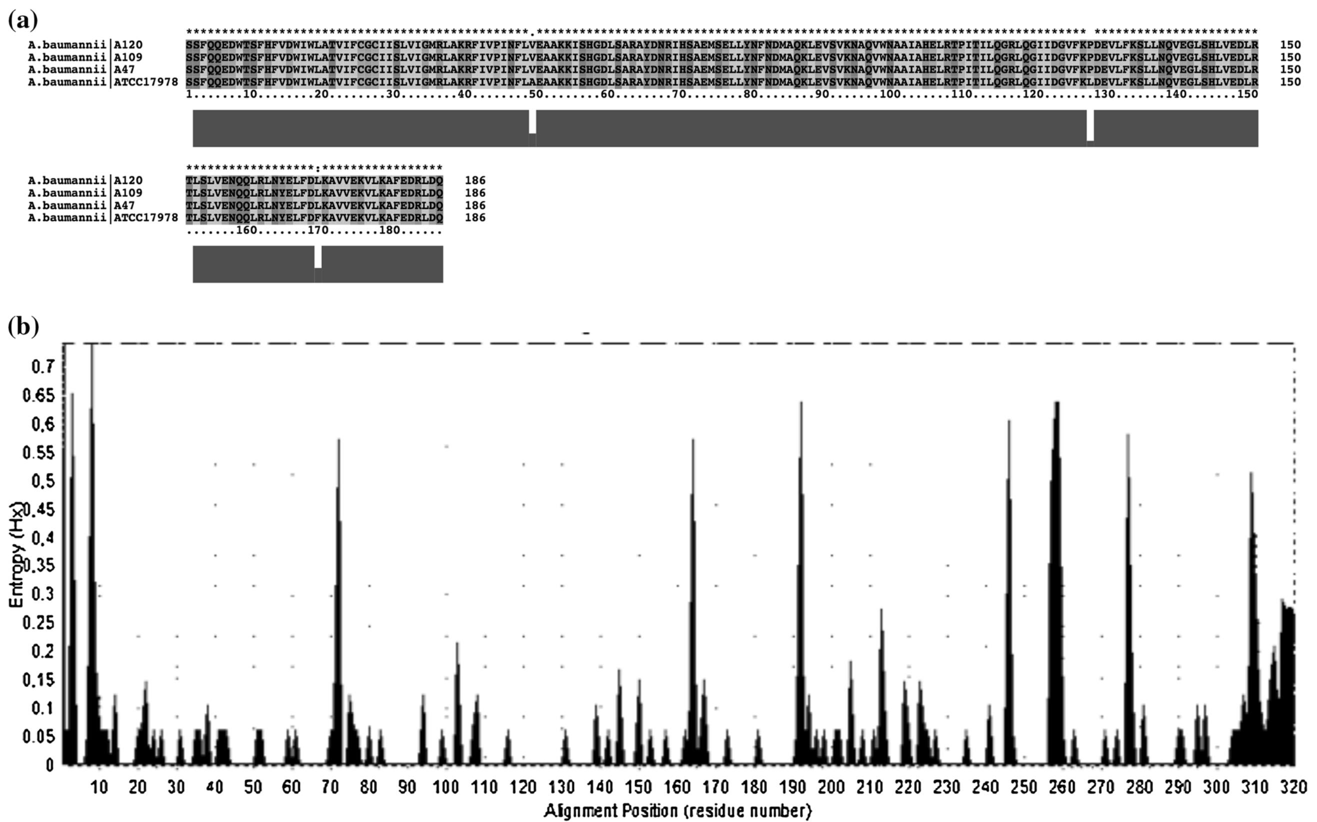

Fig. 2 **a** Amino acid sequence comparison of the AdeS sequence described in this study. The comparison was performed using the AdeS sequence of the *A. baumannii* ATCC 17978 strain as reference. The *stars* show identity among the amino acid sequences. **b** Histogram of AdeS sequence variability. The height of each *bar* indicates the degree of amino acid sequence variation from the consensus amino acid residue at that location, measured by the Shannon entropy for all AdeS proteins in the 17 *A. baumannii* clinical isolates analyzed in this study

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