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ORIGINAL PAPER

Amplification of a Single-locus Variable-number Direct Repeats with Restriction Fragment Length Polymorphism (DR-PCR/RFLP) for Genetic Typing of *Acinetobacter baumannii* Strains

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Abstract

In search of an effective DNA typing technique for *Acinetobacter baumannii* strains for hospital epidemiology use, the performance and convenience of a new target sequence was evaluated. Using known genomic sequences of *Acinetobacter baumannii* strains AR 319754 and ATCC 17978, we developed single-locus variable-number direct-repeat analysis using polymerase chain reaction-restriction fragment length polymorphism (DR-PCR/RFLP) method. A total of 90 *Acinetobacter baumannii* strains isolated from patients of the Clinical Hospital in Bydgoszcz, Poland, were examined. Initially, all strains were typed using macrorestriction analysis of the chromosomal DNA by pulsed-field gel electrophoresis (REA-PFGE). Digestion of the chromosomal DNA with the ApaI endonuclease and separation of the fragments by PFGE revealed 21 unique types. Application of DR-PCR/RFLP resulted in recognition of 12 clusters. The results showed that the DR-PCR/RFLP method is less discriminatory than REA-PFGE, however, the novel genotyping method can be used as an alternative technique for generating DNA profiles in epidemiological studies of intra-species genetic relatedness of *Acinetobacter baumannii* strains.

Key words: *Acinetobacter baumannii*, DR-PCR/RFLP, REA-PFGE, repeated sequences, nosocomial infections

Introduction

Acinetobacter spp. are aerobic Gram-negative organisms widely distributed in the soil and water of natural environments (Baumann, 1968) and are also important nosocomial pathogens. *Acinetobacter* outbreaks involving multidrug-resistant strains have occurred worldwide (Bergogne-Berezin, 2001; Bergogne-Berezin and Towner, 1996; Dijkshoorn *et al*., 1993; Spence *et al*., 2004; Dolzani *et al*., 1995; Paul *et al*., 2005; Wright 2005; Zapor and Moran, 2005). In hospitalized patients, *Acinetobacter baumannii* frequently colonizes the skin and upper respiratory tract and has been isolated from human sputum, blood, urine, and feces (Baltimore *et al*., 1989; Rosenthal and Tager, 1975; Al-Khoja and Darrell, 1979). They are often resistant to commonly used antibiotics and may form a reservoir of antibiotic resistance genes, particularly in hospital environments.

Understanding the fundamental mechanisms underlying *Acinetobacter* infections, including the original sources of the infecting organisms, their clonality, and geographical spread, is an important requirement for the development of appropriate infection control measures. In epidemiological studies of *A. baumannii* infections, many phenotyping and genotyping methods were developed. To date, several methods for the genotyping of *A. baumannii* isolates have been reported. Genotyping allows investigation of clonal spread and can be used to identify the source of the original infection. Traditional *Acinetobacter* strain typing methods include serotyping (Traub, 1989), multilocus enzyme electrophoresis (Seltmann *et al*., 1995), and DNA-based methods, including repetitive extragenic palindromic sequence-based PCR (Bou *et al*., 2000; Misbah *et al*., 2004; Huys *et al*., 2005), amplified ribosomal DNA restriction analysis (ARDRA), pulsed-field gel electrophoresis (PFGE) (Gouby *et al*., 1992; Seifert

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and Gerner-Smidt, 1995), amplified fragment length polymorphism (AFLP) (Koeleman *et al*., 1998), ribotyping (Vaneechoutte *et al*., 1995; Ibrahim *et al*., 1997), and multilocus sequence typing (MLST) (Ecker *et al*., 2006).

Many laboratories are searching for a method that can provide the appropriate level of discriminatory power and is relatively rapid and cheap, especially for large-scale population studies.

In the present study we report the application of single-locus variable-number direct-repeats analysis (DR-PCR/RFLP) for the study of intra-species genetic relatedness of *A. baumannii*. Results from this genotyping assay and REA-PFGE analysis were compared.

Experimental

Materials and Methods

Bacterial strains. A total of 90 *A. baumannii* strains isolated from various clinical specimens of patients from University Hospital of Dr A. Jurasz in Bydgoszcz during 2003–2006, and reference strain *A. baumannii* CIP 70.34^{T} (ATCC 19606^{T}) were examined. Species identification and biochemical characterization were performed with the biochemical profile index procedure ID GN (bioMérieux, France). The antimicrobial susceptibility test was performed according to standardized disc diffusion Kirby-Bauer method according to CLSI recommendations.

REA-PFGE. Initially, all isolates were tested for epidemiological relationships using REA-PFGE. PFGE was performed with the Bio-Rad's Instruction Manual and Application Guide. *A. baumannii* isolates were grown overnight (18 h at 35°C) on Columbia Agar with 5% sheep blood (bioMérieux, France). Briefly, for the preparation of plugs 200 µl of the bacterial cell suspension was gently mixed with 300 ml of 2% low melting point agarose (BioRad) in a 40°C temperature block, transferred to plug molds and allowed to solidify at 4°C. For cell lysis and protein digestion, the plugs were washed with Wash Buffer (20 mM Tris, pH 8.0, 50 mM EDTA), placed in Proteinase K Reaction Buffer (100 mM EDTA, pH 8.0, 0.2% sodium deoxycholate, 1% sodium lauryl sulfate, 1 mg/ml Proteinase K (A&A Biotechnology, Poland) and incubated for 3 h at 55°C. After that, plugs were washed four times with Wash Buffer for 30–60 min at room temperature on a rocker. DNA was digested overnight at 37°C with 20 U of ApaI (MBI Fermentas, Lithuania) for 1/3 plug, and separated on 1.2% agarose gel using the Mapper system (BioRad). Electrophoresis was carried out at 200 V in a buffer containing 0.5×TBE buffer (45 mM Tris, pH 8.0, 45 mM boric acid, 1 mM EDTA) with pulses from 2 to 20 s, for 24 h. Gels were stained with ethidium bromide and photographed under UV light, with use of the GEL DOC 2000 (BioRad). The interpretation of the banding patterns was carried out visually according to the Tenover guidelines (Tenover *et al*.,1995).

Direct repeat locus identification. The currently known *A. baumannii* strains AR 319754 (US patent 6562958-A, sequence number 2304) and ATCC 17978 nucleotide sequences were scanned for direct repeats by using the Clone Manager 4 and Tandem Repeats Finder program (http://tandem.bu.edu/trf/trf.html).

DR-PCR/RFLP. Genomic material from cultured samples was prepared with the Genomic mini (A&A Biotechnology, Poland) according to the manufacturer's protocols. All PCRs were assembled in 25-µl reaction mixtures using Gene Amp System 2400 thermocycler (Perkin Elmer). The PCR mix consisted of 1 U of RUN DNA polymerase (A&A Biotechnology, Poland), reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 20 mM Tris, pH 8.5), 2 mM $MgSO_4$, 2 mM of each deoxynucleoside triphosphate, 25 pM of each primer (Tan1 and Tan2, Table I) and 50 ng/µl of template DNA. The following PCR conditions were used to generate the amplicons: 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 68 °C for 1 min, 72°C for 2 min, and 72°C for 10 min for final extension. Amplified products were electrophoresed on 1.5% agarose gels stained in ethidium bromide. Images of the gels were analyzed using a Versa Doc Imaging System version 1000 (BioRad).

Table I
PCR primers used in this study

Primer	Sequence
Tan1	5'AGAAGAGGCTCGTGAAGCTGGTGC
Tan2	5'GCATCGCGTTTTTGATTACGAGAGTTCTGG
Rep2	5'GCCGTGCTGCACATGCCAG

The PCR products were digested with the HaeIII, SsiI (MBI Fermentas, Lithuania) and Hpy99I (BioLabs, New England) endonucleases according to manufacturer's recommendations. Digested products together with molecular weight marker (Fermentas M23; MBI Fermentas, Lithuania) were electrophoresed on 12% polyacrylamide gels with TBE buffer. Images of the ethidium bromide stained gels were analyzed using a Versa Doc Imaging System version 1000 (BioRad).

Sequencing. Based on the known *A. baumannii* nucleotide sequence the primers Tan2 and Rep2 (Table I) were designed and synthesized. PCR was performed in the reaction solution consisted of 50 ng/µl of *A. baumannii* DNA, 20 pM of each primer, 2 mM of each dNTPs, reaction buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 20 mM Tris, pH 8.5), 2 mM $MgSO_4$ and 2 U of WALK DNA polymerase (A&A Biotechnology). The thermal profile consisted of an initial denaturation step at 94°C for 1 min, fol-

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lowed by 35 cycles of a 94°C for 1 min, 66°C for 1 min, and 72°C for 2 min. At the end of amplification mixture was subjected to the final extension at 72°C for 10 min. The amplified products were isolated from an agarose gel bands using Gel-Out Kit (A&A Biotechnology). The purified fragments were ligated into pJET1/blunt vector (Gene JET™ PCR Cloning Kit, MBI Fermentas, Lithuania). DNA inserts of obtained recombinant plasmids were sequenced using automatic 310 Applied Biosystems sequencing system.

Results and Discussion

Typing by REA-PFGE. The entire collection of 90 *A. baumannii* isolates was initially analyzed by REA-PFGE (Fig. 1). Clustering yielded 21 different REA-PFGE types, designated A to W, among all isolates studied. The incidence of the types identified is shown in Table II. The six most common REA-PFGE types, represented by 8 or more isolates each, altogether comprised 68 *A. baumannii* isolates (75.5%) when one isolate of a given type per patient was considered. These were types A (14 isolates), B (13 isolates), C (13 isolates), D (10 isolates), E (10 isolates), and F (8 isolates). Five other REA-PFGE types grouped from 2 to 3 isolates each and altogether included 12 isolates (13.3%). Finally, the remaining 10 types were unique among the isolates studied (11.1%).

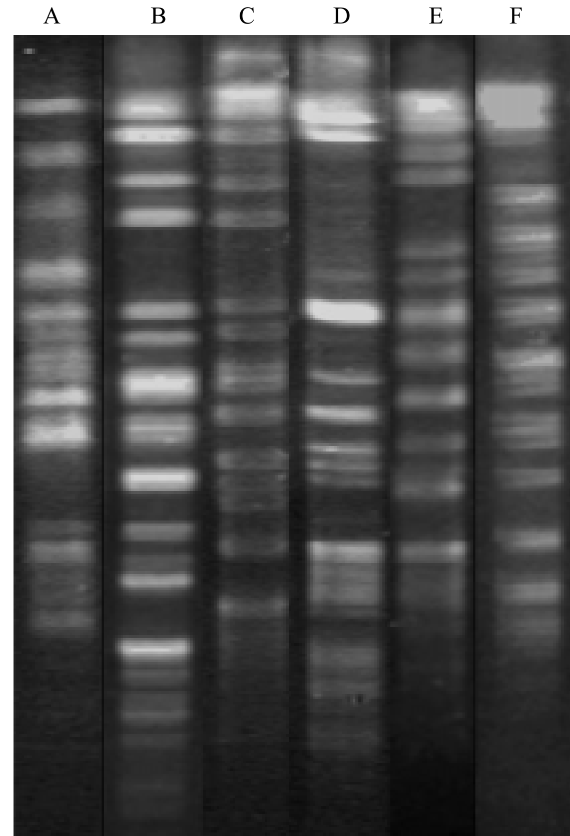

Fig. 1. PFGE profiles for main genotypes of *A. baumannii* isolates. Names of genotypes refer to genotypes shown in Table II. Chromosomal DNA was digested with ApaI, and the fragments were fractionated in 1.2% agarose gel.

Table II
REA-PEGE typing results of *A. baumannii* strains

REA-PEGE types	Total number of isolates	Year of isolation/Number of isolates			
		2003	2003	2003	2003
A	14	0	0	9	5
B	13	0	0	2	11
C	13	5	5	3	0
D	10	0	4	6	0
E	10	0	0	9	1
F	8	0	5	3	0
G	3	1	2	0	0
H	3	3	0	0	0
I	2	0	0	2	0
J	2	2	0	0	0
K	2	0	0	2	0
L	1	1	0	0	0
M	1	0	1	0	0
N	1	0	1	0	0
O	1	1	0	0	0
P	1	1	0	0	0
R	1	0	0	0	1
S	1	1	0	0	0
T	1	0	0	1	0
U	1	0	0	0	1
W	1	0	1	0	0
Total	90	15	19	37	19

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GCCGTGCTGCACATGCCAGTGGTAACGCACATGAATTTACCTCAGAAGAGGCTCGTGAAGCTGGTGCTTTAA
GTCATAAAAACGATGATCGTAATGGTCGTGGTCGCAGCCGTTATGATGACGACGAAGATGATGACGGTGGCC
GTTCAAGTGGTCGAGGCCGTGGCCGCAGTCGTTATGATGATGACGACGAAGATGATGATCGCGGTCGCTCAG
GCGGTCGTGGCCGTGGTCGCAATCGTGATGATGACGACGAAGGTGATGATCGCGGTCGCTCAGGTGGCCGAG
GCCGTGGTCGCAGCCGTGATGATGACGATGAAGATGATGATCGCGGTCGTTCAGGTGGCCGAGGTCGTGGTC
GCAGCCGCCGTGATGACGACGATGAAGATGATGATCGCGGTCGTTCAGGCGGTCGAGGTCGTGGCCGCAGCC
GCCGTGATGACGACGATGAAGATGATGATCGCGGTCGCTCAGGTGGCCGAAGTCGTGGCCGCAGCCGCCGTG
ATGACGACGATGAAGATGATGATCGTGGCCGTTCAGGTGGCCGAGGCCGTGGTCGCAGCCGTTATGATGACG
ACGATGAAGATGATGACCGTGGCCGTTCAGGCGGTCGAGGCCGTGGCCGCAGCCGTTATGATGACGACGATG
AAGATGATGACCGTGGCCGTTCAGGTGGCCGAGGCCGTGGTCGCAGCCGTCGTGACGATGACGACGAAGATG
ATGATCGTGGCCGTTCAGGTGGCCGAGGCCGTGGTCGCAGCCGTTATGATGACGACGATGAAGATGATGACC
GTGGCCGTTCAGGTGGCCGAGGCCGTGGTCGCAGCCGTCGTGATGATGATGACGAAGATGATGATCGCGGTC
GTTCAGGTGGTCGAGGCCGTGGCCGCAGTCGTTATGATGATGACGATGAAGATGATGATCGTGGTCGTTCAG
GTGGCCGAGGCCGTGGTCGCAGCCGTCGTGATGACGATGACGACGATGATGACCGCCGTGGCCGTTCAGATG
GTCGTGGCCAGAACTCTCGTAATCAAAAACGCGATGC
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Fig. 2. Nucleotide sequence of *A. baumannii* DNA fragment containing direct repeat sequences (in grey). Nucleotide sequences of Tan1, Tan 2 and Rep2 are underlined (see Table I).

The *A. baumannii* clone of REA-PFGE type A has been the most prevalent clone of the species in hospital. It made its first appearance among the isolates studied in 2005 (9 isolates) and also was identified in 2006 (5 isolates). It was constantly observed in various wards of the hospital. The second most prevalent *A. baumannii* clone was REA-PFGE type B. The earliest isolate of the type B clone in the analysis was identified in 2005 (2 isolates) and was isolated until the end of the study in 2006 (11 isolates). The first isolate of the REA-PFGE type C clone (5 isolates) recovered in the study was detected in 2003 and was dominated type in the hospital during that year. This type was also dominated 1 year later. Isolates of the REA-PFGE type E clone (10 isolates) were first recorded in hospital in 2005 (9 isolates), and was detected in 2006. Twenty-four isolates were found to represent sporadic REA-PFGE types.

Typing by DR-PCR/RFLP. The analysis of *A. baumannii* sequenced genome of AR 319754 and ATCC 17978 strains revealed the presence of direct repeat DNA sequences, located at one position (Fig. 2). This sequence comprises two 19-bp direct repeats with different nucleotides at 8 position and ten 22-bp direct repeats with different nucleotides at 5, 8, 11, 14, 16 and 17 positions and two 25-bp direct repeats with different nucleotides at 8 and 14 positions. Direct repeat sequences are interspersed with 41–47 bp polymorphic sequences (Fig. 2). Analysis of this repeat region was included to determine its value for genotyping of *A. baumannii* strains. Based on this sequence oligonucleotide sequences of two primers for the PCR were designed (Tan1 and Tan2, Table I). A total of 90 clinical *A. baumannii* strains and reference strain were used in this study. The Tan1 and Tan2 primers enabled amplifications of specific regions from all strains tested giving PCR products with length of approximately 1000 bp.

The computer analysis of the *A. baumannii* direct repeat DNA sequences was performed to determine the most discriminative restriction enzyme for RFLP analysis based on restriction maps.

Due to the data obtained we could choose three restriction enzymes – HaeIII, Hpy99I and SsiI – as the most discriminating. The RFLP analysis with the HaeIII, Hpy99I and SsiI could distinguish 6, 10 and

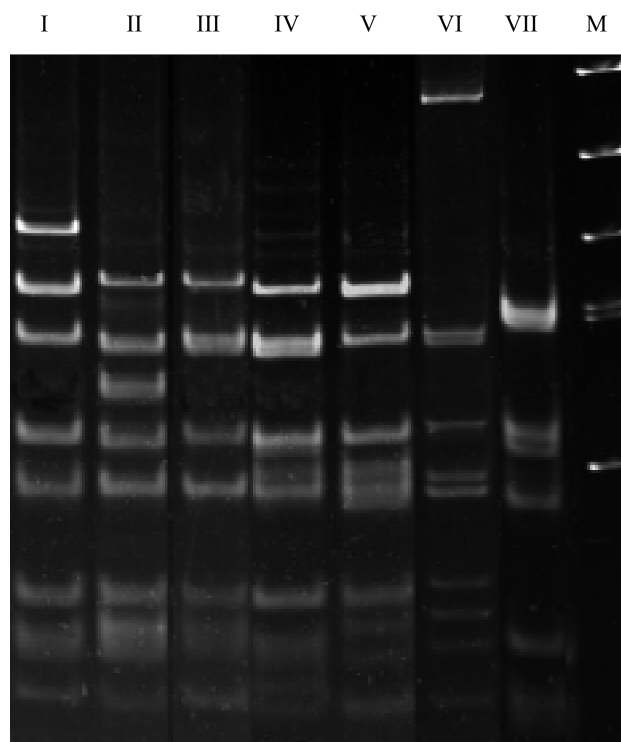

Fig. 3. DR-PCR/RFLP patterns of the *A. baumannii* isolates (representative results) of all types obtained by SsiI restriction enzyme. The lane designated M contains the molecular mass marker (242, 190, 147, 111, 110 and 67 bp). The DNA fragments were electrophoresed in 12% polyacrylamide gel. In the panels the genotype names are given above each lane.

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Table III
DR-PCR/RFLP typing results for clinical and reference *A. baumannii* strains

Restriction pattern			Total restriction pattern	DR-PCR/RFLP types	Number of strains	REA-PFGE types
HaeIII	Hpy99I	SsiI(AciI)				
A	A	A	AAA	I	40	B(13) + A(10) + D(10) + E(7)
B	B	B	BBB	II	18	F(7) + A(3) + G(3) + other(5)
A	C	B	ACB	III	3	I(2) + J + (1)
A	D	B	ADB	IV	5	C(3) + I(1) + H(1)
A	E	C	AEC	V	9	C(8) + L(1)
C	F	D	CFD	VI	2	M(1) + K(1)
D	D	E	DGE	VII	1	O(1)
A	E	B	AEB	VIII	2	J(1) + C(1)
A	D	C	ADC	IX	3	H(2) + F(1)
E	H	F	EHF	X	1	W(1)
A	I	B	AIB	XI	3	C(1) + E(1) + U(1)
A	D	A	ADA	XII	3	E(2) + A(1)
F	J	G	FJG	XIII *A. baumannii* CIP 70.34^T (ATCC 19606^T)		
6	**10**	**7**	**12**		**90**	

7 pattern types, respectively, among 90 strains tested. The combination of HaeIII, Hpy99I and SsiI restriction patterns gave 12 composite pattern types, designated I to XII (Table III). Representative results of RFLP analysis with the SsiI are shown in Fig. 3. The largest cluster I of DR-PCR/RFLP patterns consisted of 40 strains and analysis showed that isolates with REA-PFGE types A, B, D and E were most related and belonging to this cluster.

Here we show the evaluation of a novel genotyping method based on single-locus variable-number direct-repeat analysis using polymerase chain reaction-restriction fragment length polymorphism (DR-PCR/RFLP) for epidemiological studies of *A. baumannii* clinical strains. DR-PCR/RFLP method was able to identify the most dominant genotypes among clinical strains examined (genotypes I and II, Table III). This study shows much lower discriminatory power of DR-PCR/RFLP method in comparison to the REA-PFGE. However, the DR-PCR/RFLP method is cheaper, faster, and easier to perform than REA-PFGE.

Sequencing data. For four *A. baumannii* strains belonging to the most prevalent DR-PCR/RFLP type I, the PCR products with Tan2 and Rep2 primers (Table I) were obtained, cloned into pJET1/blunt Vector and sequenced. The obtained DNA sequences were used to identify and compare theirs direct repeats sequences (Fig. 4). Multiple sequence alignment was generated by using the program ClustalX. The results were prepared using the editor program Gendoc (copyright Karl Nicholas). Exactly the same sequences were obtained for four tested strains of type I. The nucleotide sequence for one of them was deposited in the GenBank database under accession number DQ 785810. A 63-bp deletion with one direct repeat was revealed in comparison to *A. baumannii* sequenced genome of AR 319754. There is also a small heterogeneity between tested strains of type I and *A. baumannii* sequenced genome of AR 319754 in rest of the sequence (96.1% homogeneity).

For further analysis PCR product of clone type II was cloned into pJET1/blunt vector and sequenced

Table IV
Simulated and real restriction patterns obtained from restriction maps for HaeIII, Hpy99I and SsiI enzymes

Sequences	DR-PCR/RFLP (group)	HaeIII	Hpy99I	SsiI
DQ785810 2378 2360 2359	I	99, 78, 60, 57, 54, 51, 48, 42, 24, 18, 12, 6	299, 247, 133, 73, 68, 66, 41, 12	170, 124, 102, 78, 66, 56, 38, 34, 33, 32, 28, 18, 16, 12
EU009127	II	99, 78, 60, 57, 54, 51, 48, 42, 24, 18, 10, 6	320, 198, 132, 71, 68, 66, 41, 40	126, 102, 96, 94, 81, 66, 38, 34, 31, 18
AR319754		99, 78, 66, 60, 57, 54, 51, 48, 42, 30, 18, 12, 6	132, 126, 83, 66, 64, 63, 55, 17, 14	236, 124, 110, 102, 63, 56, 51, 38, 34, 32, 28, 16, 12, 6

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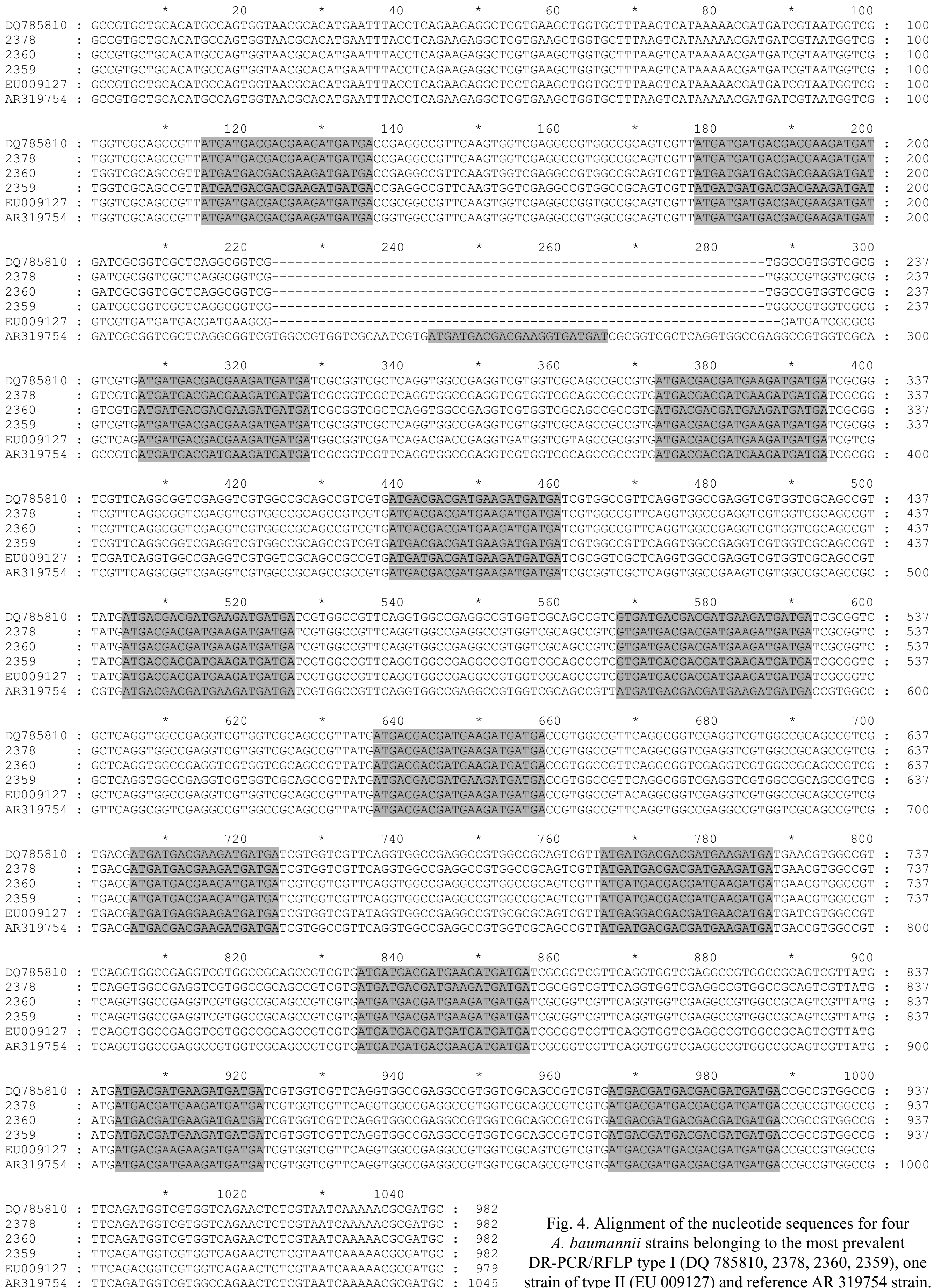

Fig. 4. Alignment of the nucleotide sequences for four *A. baumannii* strains belonging to the most prevalent DR-PCR/RFLP type I (DQ 785810, 2378, 2360, 2359), one strain of type II (EU 009127) and reference AR 319754 strain.

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(Fig. 4). The obtained nucleotide sequence was deposited in the GenBank database under accession number EU 009127. A 65-bp deletion with one direct repeat was revealed in comparison to *A. baumannii* sequenced genome of AR 319754. The results shown in that analysis indicate also heterogeneity between type I, II and *A. baumannii* AR 319754 sequences (93.7% homogeneity between type I and II, and 91.1% between type II and *A. baumannii* AR 319754).

The computer analysis of the obtained nucleotide sequences revealed that the restriction patterns determined from the sequencing data for three restriction enzymes (HaeIII, Hpy99I and SsiI) were with accordance to restriction patterns obtained using DR-PCR/RFLP method (Table IV).

The present study clearly revealed that the RFLP profiles obtained with restriction enzymes used might be useful for differentiation of *A. baumannii* strains. The use of additional enzymes might allow a higher degree of discrimination between isolates and may be regarded as an auxiliary method in relation to REA-PFGE for epidemiological studies.

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