Validation of partial *rpoB* gene sequence analysis for the identification of clinically important and emerging *Acinetobacter* species

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Bacteria belonging to the genus *Acinetobacter* are ubiquitous in soil and water. Only a few species, including *Acinetobacter baumannii*, and the unnamed *Acinetobacter* genomic species (gen. sp.) 3 and 13TU, which together with the soil organism *Acinetobacter calcoaceticus* are combined in the *A. calcoaceticus*–*A. baumannii* (Acb) complex, have been recognized as important nosocomial infectious agents. The ecology, epidemiology and pathology of most species are not yet well established. Lack of practical and accurate methods limits routine identification of clinical isolates and thus hampers precise identification of those of the Acb complex and other *Acinetobacter* species of possible clinical significance. We previously identified a 350 bp highly variable zone on the *rpoB* gene which appeared to be a promising target for rapid molecular identification. In the present study, we validated this method for accuracy on a collection of reference strains belonging to *A. calcoaceticus* (5 strains), *Acinetobacter* gen. sp. 3 (29 strains), *A. gen. sp. 13TU* (18 strains), *A. baumannii* (30 strains) and one strain each of *A. radioresistens*, *A. gen. sp. 15TU*, *A. gen. sp. 10*, *A. gen. sp. 11*, *A. gen. sp. 'between 1 and 3'* and *A. gen. sp. 14TU=13BJ*. This represents the largest analysis to date that compares a large number of well-identified strains of the Acb complex to assess the intra- and interspecies variation within this complex. All were correctly identified with 98.9–100% intraspecies relatedness based on partial *rpoB* sequence analysis. We then applied this tool to identify 99 *Acinetobacter* clinical isolates from four public hospitals in Marseille, France. All isolates could easily be identified to species as they were separated into 13 species sequence types with a sequence variance of 0–2.6% from their respective type strains. Of these 99 isolates, 10 were *A. haemolyticus*, 52 were *A. baumannii*, 27 were *A. gen. sp. 3*, 5 were *A. schindleri*, 1 was *A. lwoffii*, and 1 was *A. gen. sp. 13TU*. Three were provisionally identified as *A. gen. sp. 9*. This is the first work to identify all specimens of a set of clinical *Acinetobacter* isolates at species level using *rpoB* sequence analysis. Our data emphasize the recognition of *A. schindleri* as an emerging cause of *Acinetobacter*-related infection and confirm that *A. gen. sp. 3* is the second most commonly isolated *Acinetobacter* species after *A. baumannii* in patients.

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INTRODUCTION

Bacteria belonging to the genus *Acinetobacter* are strictly aerobic, oxidase-negative, non-fermenting, non-motile,

Abbreviations: Acb complex, *A. calcoaceticus*–*A. baumannii* complex; AFLP, amplified fragment length polymorphism; gen. sp., genomic species; PRBS, partial *rpoB* sequencing; seqtype, sequence type.

Two supplementary tables are available with the online version of this paper, showing seqtype results of the training set of 107 *Acinetobacter* strains by PRBS analysis, and details of the identification of 99 clinical isolates by PRBS analysis.

Gram-negative, coccobacillary organisms. Over the last 20 years, particular *Acinetobacter* species have emerged as opportunistic pathogens that are associated with severe hospital-acquired infections including pneumonia, blood-stream infections and wound infections (Bergogne-Berezin & Towner, 1996; Dijkshoorn *et al.*, 2007; Joly-Guillou, 2005). A matter of concern is the ability of multidrug-resistant *Acinetobacter* strains to spread epidemically on wards with critically ill patients. The avalanche of reports in recent years describing outbreaks involving these organisms emphasizes the global dimension of this problem. A

recent, striking manifestation of acinetobacters is their association with wound infections in US and UK soldiers injured in Iraq (Davis *et al.*, 2005).

The taxonomy of the genus *Acinetobacter* is complex. Currently, 20 validly named species have been described, while 12 additional genomic species with as yet provisional designations have been identified by DNA–DNA hybridization (Dijkshoorn *et al.*, 2007). Unambiguous identification of acinetobacters at the species level is problematic. Specifically designed phenotypic methods have been found useful for most, but not all, currently known *Acinetobacter* species (Bouvet & Grimont, 1987; Gerner-Smidt *et al.*, 1991; Kampfer *et al.*, 1993). Commercial systems used in diagnostic bacteriology are also not satisfactory for *Acinetobacter* species identification (Bernards *et al.*, 1995, 1996; Dortet *et al.*, 2006). In order to solve this problem, several genotypic identification methods have been explored. Of these, DNA polymorphism analysis based on selective amplification of restriction fragments (AFLP analysis) and amplified 16S rDNA restriction analysis (ARDRA) library identification have been validated with large sets of reference strains of all *Acinetobacter* species (Dijkshoorn *et al.*, 1998; Janssen & Dijkshoorn, 1996; Nemec *et al.*, 2001), but these methods are used in only a few *Acinetobacter* reference laboratories and are not amenable to use in routine laboratories.

Acinetobacter strains from clinical infections and outbreaks are most frequently identified as *Acinetobacter baumannii*. This species is genetically closely related and phenotypically similar to two other clinical species, *Acinetobacter* genomic species (gen. sp.) 3 and 13TU, and to *Acinetobacter calcoaceticus*, a soil organism. This close similarity led to the proposal that these species should be lumped together in the so-called *A. calcoaceticus*–*A. baumannii* (Acb) complex (Gerner-Smidt *et al.*, 1991). However, numerous clinical reports identifying clinical *Acinetobacter* isolates as *A. baumannii* or as belonging to the Acb complex are based on identification methods that do not allow for unambiguous species identification. Consequently, the epidemiology and clinical significance of the different species within the Acb complex are not yet fully understood. Furthermore, the ecology and clinical role of most other *Acinetobacter* species, including the recently described species *Acinetobacter ursingii*, *Acinetobacter schindleri* and *Acinetobacter parvus*, need to be assessed further.

In a previous study (La Scola *et al.*, 2006), as an alternative to previously designed molecular identification methods, we sequenced the complete *rpoB* gene, which encodes an RNA polymerase β -subunit gene, and identified four hyper-variable zones (two of the zones are on the *rpoB* gene, sizes 350 and 450 bases; the other two are flanking spacers at both ends of the *rpoB* gene, i.e. *rplL*–*rpoB*, 301–310 bases, and *rpoB*–*rpoC*, 86–177 bases). We then developed a molecular method (referred to herein as PRBS, for partial *rpoB* sequencing) by designing primers to amplify these four short fragments for rapid and accurate

identification of *Acinetobacter* species (La Scola *et al.*, 2006). One of these fragments, namely the 350 bp zone 1, was predicted to be the most useful for identification of *Acinetobacter* species. In the present study, we validated the PRBS method, which consists of PCR and sequencing of a 350 bp partial *rpoB* gene, using one collection set of a large number of well-identified isolates, including those of the Acb complex. Later, as a demonstration of the applications of this technique in clinical microbiology laboratories, we identified a collection of clinical isolates of *Acinetobacter* in the four public hospitals of Marseille, France.

METHODS

Bacterial isolates. The collection set was composed of strains available in three culture collections and included a total of 113 strains of *Acinetobacter* species or genomic species; it was obtained from the *Acinetobacter* reference collection of the Leiden University Medical Centre (LUMC), Leiden, the Netherlands [91 cultures], from the Culture Collection of the Institute Pasteur (CIP), Paris, France [21 cultures] and from the Collection de Souches de l'Unité des Rickettsies (CSUR) [1 culture]. An overview of the collection set is given in Supplementary Table S1, available with the online version of this paper. The LUMC strains (with designations LUH or RUH) comprised mostly human clinical isolates, many of which had been used in previous studies (Bouvet & Jeanjean, 1989; Dijkshoorn *et al.*, 2005; Janssen & Dijkshoorn, 1996; Nemec *et al.*, 2007; Tjernberg & Ursing, 1989). The strains identified by well-established identification methods, e.g. AFLP analysis (LUMC strains) (Nemec *et al.*, 2001) and/or by DNA–DNA hybridization (Bouvet & Grimont, 1987; Bouvet & Jeanjean, 1989; Tjernberg & Ursing, 1989), were used as reference strains for validation of our identification method. Species identification by AFLP analysis was obtained by comparing genomic fingerprints of strains resulting from selective amplification of restriction fragments to a library of fingerprints of strains all identified by DNA–DNA hybridization (Nemec *et al.*, 2001). A similarity level of $\geq 50\%$ was used as species cut-off level.

The clinical set contained 99 isolates from various clinical specimens obtained between February 2001 and March 2005 in the four public hospitals of Marseille, a city in southern France. These isolates were obtained from blood [47], an autologous blood unit [1], bone and intra-articular material [44], ascites fluid [2], cerebrospinal fluid [3], soft tissue [2] and a vascular prosthesis [1]. All isolates were grown on Columbia agar plates with 5% sheep blood (Trypticase soy agar; bioMérieux) at 35 °C for 24–48 h, and the bacterial colonies were used for DNA extraction.

DNA extraction, PCR and sequencing. Bacterial DNA of the LUMC reference strains was extracted by the method of a previous study (Boom *et al.*, 1990). DNA from other strains was extracted from a heavy suspension of strains, using the QIAamp blood kit (Qiagen), according to the manufacturer's recommendations. PCRs incorporated previously described primers for zone 1 amplification (La Scola *et al.*, 2006). The PCR mixtures (50 μ l) contained 2 μ l extracted DNA, 2.5×10^{-2} U *Taq* polymerase μ l $^{-1}$; $1 \times$ *Taq* buffer; 1.8 mM MgCl₂ (Gibco-BRL Life Technologies); dATP, dCTP, dTTP and dGTP (Boehringer Mannheim) at concentrations of 200 μ M each; and each primer, Ac696F (TAYCGYAAAGAYTTGAAAGAAG) and Ac1093R (CMACACCYTTGTTMCCRTGA) (Eurogentec) at a concentration of 0.2 μ M. The PCR mixtures were subjected to 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 52 °C for 30 s, and extension at 72 °C for 1 min. Each amplification programme began with a denaturation step at 94 °C for 2 min and ended with a

Table 1. Seqtype results of the tested *Acinetobacter* strains by partial *rpoB* gene sequence analysis

Seqtype	Genomic species	No. of strains		Similarity with type strain (%)	GenBank accession no.
		Collection (113)	Clinical (99)		
G1	<i>A. baumannii</i>	16	44	98.3	EU445581
G2	<i>A. baumannii</i>	1		98.0	EU445595
G3	<i>A. baumannii</i>	3		98.6	DQ207459
G4	<i>A. baumannii</i>	1		98.3	DQ207467
G5	<i>A. baumannii</i>	1		98.3	EU445598
G6	<i>A. baumannii</i>	4	3	98.0	EU445599
G7	<i>A. baumannii</i>	2		98.3	DQ207468
G8	<i>A. baumannii</i>	1		98.6	DQ207461
G9	<i>A. baumannii</i>	2		98.9	DQ207457
G10	<i>A. baumannii</i>	7	1	98.3	DQ207455
G11	<i>A. baumannii</i>	4	3	99.1	DQ207451
G12	<i>A. baumannii</i>	3		100	EU445609
G13	<i>A. baumannii</i>	3		99.7	DQ207462
G14	<i>A. baumannii</i>	1		99.4	DQ207456
G15	<i>A. baumannii</i>		1	98.6	EU445676
G16	Gen. sp. 13TU	1			EU445612
G17	Gen. sp. 13TU	9			EU445621
G18	Gen. sp. 13TU	3			EU445624
G19	Gen. sp. 13TU	5			EU445629
G20	Gen. sp. 13TU		1		EU445680
G21	Gen. sp. 3	1			EU445630
G22	Gen. sp. 3	6	26		EU445631
G23	Gen. sp. 3	2			EU445637
G24	Gen. sp. 3	1			EU445639
G25	Gen. sp. 3	1			EU445640
G26	Gen. sp. 3	13			EU445653
G27	Gen. sp. 3	2			EU445654
G28	Gen. sp. 3	1			EU445656
G29	Gen. sp. 3	1			EU445657
G30	Gen. sp. 3	1			EU445658
G31	Gen. sp. 3	1			EU445659
G32	Gen. sp. 3		1	99.4	EU445679
G33	<i>A. calcoaceticus</i>	1		99.4	EU445660
G34	<i>A. calcoaceticus</i>	3		99.7	EU445663
G35	<i>A. calcoaceticus</i>	1		98.6	EU445664
G36	Gen. sp. 10	1			EU445665
G37	Gen. sp. 11	1			EU445666
G38	<i>A. radioresistens</i>	1		99.1	EU445667
G39	'Between 1 and 3'	1			EU445668
G40	'Close to 13TU'	1			EU445669
G41	Gen. sp. 14TU/13BJ	1			EU445670
G42	Gen. sp. 15TU	1			EU445671
G43	Gen. sp. 14BJ	1			EU445672
G44	Gen. sp. 15BJ	1			EU445673
G45	Gen. sp. 16	1			EU445674
G46	Gen. sp. 17	1			EU445675
G47	<i>A. haemolyticus</i>		10	100	DQ207484
G48	<i>A. schindleri</i>		3	100	DQ207490
G49	<i>A. schindleri</i>		2	97.4	EU445678
G50	<i>A. lwoffii</i>		1	98.9	EU445677
G51	'Gen. sp. 9'*		3	96.6	EU445681

*As similarity is low with strain CIP 64.7 and no identification confirmatory procedure was done, identification may not be confirmed as definitive.

final elongation step at 72 °C for 7 min. PCR amplicons were purified for sequencing using the QIAquick spin PCR purification kit (Qiagen), according to the protocol of the manufacturer. Sequencing reactions were carried out with the reagents of the ABI Prism 3100 DNA sequencer (dRhod.Terminator RR Mix; Perkin-Elmer Applied Biosystems), according to the standard automated sequencer protocol.

The 16S rRNA gene sequence of a subset of collection strains in the Acb complex and some clinical strains was also determined as previously described (Drancourt *et al.*, 2004).

Analysis of sequence data. The nucleotide sequences of the *rpoB* gene fragment obtained in this study were processed into sequence data with Sequence Analysis Software (Applied Biosystems). Both strands were sequenced and partial sequences were combined into a single consensus sequence with Sequence Assembler Software. Multiple-sequence alignments between the different species were done with the CLUSTAL W program (Thompson *et al.*, 1994) on the EMBL-EBI web server (<http://www.ebi.ac.uk/clustalw>). For ambiguous positions, sequencing was repeated. The percentage similarity between all sequences was calculated by using the MEGA4 program (Tamura *et al.*, 2007). Phylogenetic trees were constructed from DNA sequences by the neighbour-joining method (Felsenstein, 1989), and bootstrap replicates were performed in order to estimate the reliability of the nodes of the phylogenetic trees. Bootstrap values were obtained from 1000 trees, generated randomly with the MEGA4 program. 16S rRNA gene sequences were analysed as described above.

RESULTS

PCR products and sequences

In the present study, 350 bp partial *rpoB* gene sequences were successfully obtained in a single attempt by PCR with Ac696F-Ac1093R primers from all collection and clinical isolates and strains of *Acinetobacter* (Table 1). All strains were classified into sequence types, a sequence type (seqtype) being defined as a unique sequence of the 350 bp partial *rpoB* gene.

The 49 *A. baumannii* cultures – 30 from the LUMC, including 7 isolates from European clones I–III (Dijkshoorn *et al.*, 1996; van Dessel *et al.*, 2004), 4 isolates from the so-called clusters A–D (Nemec *et al.*, 2007), 4 isolates from one outbreak, for the purpose of this study designated ‘outbreak 1’ (Bernards *et al.*, 2004); 18 from the CIP strains; and 1 from CSUR – were scattered into 14 different seqtypes with 98.3–100 % similarity with the type strain ATCC 19606 (Table 1 and Supplementary Table S2). Seventeen *A. gen. sp.* 13TU and CIP 103655 (received as belonging to *A. baumannii*) were grouped into four seqtypes. Thirty strains of *A. gen. sp.* 3 were separated into 11 seqtypes. Five strains of *A. calcoaceticus* were distinguished as three seqtypes with 98.6–99.4 % similarity with the type strain (RUH 2201^T, ATCC 23055^T).

Other strains belonging to *A. gen. sp.* 10, 11 and *A. radioresistens* were denoted as seqtypes 36, 37 and 38 in the present study (Table 1). Furthermore, we incorporated two additional strains, LUH 1090 (*A. gen. sp.* 15TU) and RUH 02218 (=ATCC 17905, *A. gen. sp.* 14TU=13BJ) (Tjernberg

& Ursing, 1989). RUH 2218 (=ATCC 17905), belonging to 14TU/13BJ, was shown to have 99.7 % sequence similarity with CIP 64.2 (GenBank accession no. DQ207478), confirming the identity of the subcultures. It is of note that in our previous paper (La Scola *et al.*, 2006), CIP 64.2 was erroneously described as belonging to *A. gen. sp.* 16, and CIP 70.18 as belonging to *A. gen. sp.* 13 (BJ), which corresponds to 14TU (Tjernberg & Ursing, 1989). This was due to administrative confusion in the CIP catalogue, which has now been corrected: CIP 64.2 belongs to *A. gen. sp.* 13BJ, and CIP 70.18 belongs to *A. gen. sp.* 16. GenBank data have also been corrected. LUH 1090 clustered with *A. schindleri* (similarity 90.9 %) but its low similarity and its position in phylogenetic tree confirm that it is different.

The PRBS method of determining the 350 bp *rpoB* gene partial sequence of 99 clinical isolates of *Acinetobacter* gave different sequences. They were distributed into 13 seqtypes on the basis of their sequence similarity with species type strains (Table 1). Most were identical to previously identified seqtypes. The dominant species found was *A. baumannii* (52 %; 52/99) followed by *A. gen. sp.* 3 (27 %; 27/99) and *A. haemolyticus* (10 %; 10/99). Other isolates showed close relatedness to *A. schindleri*, *A. lwoffii*, *A. gen. sp.* 13TU and *A. gen. sp.* 9 (Table 1). In the present study, the *A. schindleri* isolates were from five different patients. Three were obtained from patients with osteitis, one from blood culture and one from the cerebrospinal fluid of a patient with hospital-acquired meningitis. In the osteitis case, only one sample was taken superficially (swab from fistula). The 16S rRNA gene sequences of *A. lwoffii* and of the two *A. schindleri* that do not have 100 % similarity with reference strain confirmed correct *rpoB*-based identification (Supplementary Table S2).

DISCUSSION

In our preliminary work, we identified a 350 bp hyper-variable zone in the *rpoB* gene and used it in rapid identification of acinetobacters (La Scola *et al.*, 2006). In the present study, we validated this PRBS method on a large number of collection strains and later identified clinical isolates based on the sequence criteria we developed. The species of the Acb complex, *A. calcoaceticus*, *A. baumannii*, *A. gen. sp.* 3 and *A. gen. sp.* 13TU, are phenotypically very similar (Gerner-Smidt *et al.*, 1991). In our study, however, these species were well separated, showing an 88.3–96.9 % interspecies similarity range (Table 1). Based on 350 bp partial *rpoB* gene sequence analysis, the intraspecies similarity for Acb complex species studied so far is as follows: 98–100 % for *A. baumannii* (49 strains), 98–100 % for *A. gen. sp.* 13TU (19 strains), 98–100 % for *A. gen. sp.* 3 (30 strains) and 98.6–100 % for *A. calcoaceticus* (6 strains) (Table 1). Gerner-Smidt & Tjernberg (1993) proposed that two other closely related DNA groups, *A. gen. sp.* ‘close to 13TU’ and *A. gen. sp.* ‘between 1 and 3’, be lumped together into the Acb complex species, due to their indistinguishable phenetic

properties. In the present study, *A. gen. sp. 'close to 13TU'* (LUH01472=10090) showed 96 % sequence similarity with *A. gen. sp. 13TU*, and the *A. gen. sp. 'between 1 and 3'* shared 96.9 % sequence similarity with *A. calcoaceticus*. In the phylogenetic tree, *A. gen. sp. 'between 1 and 3'* grouped most closely to *A. calcoaceticus*, and *A. gen. sp. 'close to 13TU'* grouped most closely to 13TU. This clustering was supported by high bootstrap values (Fig. 1). The same close relatedness was also observed with data from 16S rRNA gene sequences (Table 2, Fig. 2). This emphasizes the high discriminating power of the small *rpoB* sequence as compared to the complete 16S rRNA gene sequence. Although the relatedness of species within the complex requires further investigation and testing of more strains, the topology of the phylogenetic tree suggests that *A. gen. sp. 'between 1 and 3'* most closely resembles *A. calcoaceticus* species, and *A. gen. sp. 'close to 13TU'* most closely resembles 13TU species. However, if this were true, in the Acb complex species, intraspecies similarity within each species would range from 96 to 100 %, not 98 to 100 % as observed here. Further studies are needed to resolve this issue.

By using our PRBS method, all 99 clinical isolates could easily be identified. As for two of the *A. schindleri* isolates and the *A. lwoffii* isolate *rpoB* similarity was lower than 100 %, we confirmed identification by 16S rRNA gene sequencing (Supplementary Table S2). Identification of *A. baumannii* as 52 % of the total of these isolates confirmed that it is the *Acinetobacter* species most commonly encountered in hospital infections. This observation is in agreement with a study by Seifert *et al.* (1993), who showed that the majority of isolates (72.9 %) in a large collection of 584 isolates from 420 patients belonged to *A. baumannii* (Seifert *et al.*, 1993). In the same study, *A. gen. sp. 3* (9.5 %), *A. johnsonii* (4.9 %) and *A. lwoffii* (3.6 %) were the most common species besides *A. baumannii* (Seifert *et al.*, 1993). Along with predominant *A. baumannii*, we observed that *A. gen. sp. 3* was present in significant numbers. The relatively high prevalence of *A. gen. sp. 3* in clinical specimens has been previously well documented, especially in Asia and Europe (Chu *et al.*, 1999; Dijkshoorn *et al.*, 1993; Horrevorts *et al.*, 1995; Houang *et al.*, 2001; Idzenga *et al.*, 2006; Tjernberg & Ursing, 1989). The under-

Table 2. Seqtype results of a subset of *Acinetobacter* strains in the Acb complex by partial *rpoB* and 16S rRNA gene sequence analysis

Isolate	<i>rpoB</i> seqtype	Genomic species	16S rRNA Seqtype	Comment	16S GenBank no.
RUH 0875	G1	<i>A. baumannii</i>	G1	100 % similar to CIP 70.34 ^T	
RUH 3414	G2	<i>A. baumannii</i>	G1		
LUH 8088	G3	<i>A. baumannii</i>	G2	99.9 % similar to CIP 70.34 ^T	FJ860865
RUH 1093	G5	<i>A. baumannii</i>	G1		
LUH 5687	G6	<i>A. baumannii</i>	G1		
LUH 5684	G7	<i>A. baumannii</i>	G1		
LUH 6639	G10	<i>A. baumannii</i>	G3	99.9 % similar to CIP 70.34 ^T	FJ860866
LUH 8225	G11	<i>A. baumannii</i>	G4	99.9 % similar to CIP 70.34 ^T	FJ860867
RUH 1752	G12	<i>A. baumannii</i>	G1		
LUH 7493	G13	<i>A. baumannii</i>	G1		
RUH 2627	G16	Gen. sp. 13TU	G5		FJ860868
LUH 3192	G17	Gen. sp. 13TU	G5		
LUH 3144	G18	Gen. sp. 13TU	G5		
LUH 6151	G19	Gen. sp. 13TU	G6	99.7 % similar to G5	FJ860871
LUH 6127	G21	Gen. sp. 3	G7		FJ867362
LUH 6972	G22	Gen. sp. 3	G7		
LUH 7000	G23	Gen. sp. 3	G7		
LUH 5779	G24	Gen. sp. 3	G8	99.7 % similar to G7	FJ860872
LUH 8131	G25	Gen. sp. 3	G9	99.7 % similar to G7	FJ860873
LUH 6099	G26	Gen. sp. 3	G10	99.7 % similar to G7	FJ860874
LUH 6144	G27	Gen. sp. 3	G7		
LUH 6175	G28	Gen. sp. 3	G11	99.9 % similar to G7	FJ860875
RUH 1945	G29	Gen. sp. 3	G7		
LUH 8585	G30	Gen. sp. 3	G7		
LUH 9485	G31	Gen. sp. 3	G7		
RUH 0944	G33	<i>A. calcoaceticus</i>	G12	100 % similarity to CIP 81.8 ^T	
LUH 9144	G34	<i>A. calcoaceticus</i>	G13	99.9 % similarity to CIP 81.8 ^T	FJ860876
LUH 5820	G35	<i>A. calcoaceticus</i>	G12		
LUH 1469	G39	'Between 1 and 3'	G14	99.2 % similarity to CIP 81.8 ^T	FJ860877
LUH 1472	G40	'Close to 13TU'	G15	99.3 % similarity to G5	FJ860878

Fig. 1. Dendrogram representing phylogenetic relationships between type, reference and clinical isolates of all *Acinetobacter* species. Sequences of strains determined previously (La Scola *et al.*, 2006) are indicated by their GenBank accession number in parentheses. The tree is derived from the sequences of 350 bp partial *rpoB* gene sequences. MEGA4 software (<http://www.megasoftware.net>) was used to infer the tree using the neighbour-joining method (Kimura two-parameter). The support of each branch, as determined from 1000 bootstrap samples, is indicated by the value at the node. Bootstrap values below 50 % were omitted. As previously suspected from *rpoB* sequences (La Scola *et al.*, 2006), *A. grimontii* is a so-called junior synonym of *A. junii* (Vaneechoutte *et al.*, 2008).

recognition of this species is a result of the problems encountered in correct identification using commercially available phenotypic tests (Bernards *et al.*, 1996; Dortet *et al.*, 2006).

Only one clinical isolate could be identified as *A. gen. sp.* 13TU. This isolate had 99.7 % sequence similarity with RUH 02627 of the training set. This genomic species was well discriminated from *A. baumannii* and *gen. sp. 3* and was also associated with nosocomial outbreaks (Dijkshoorn & van der Toorn, 1992; Seifert *et al.*, 1995; van Dessel *et al.*, 2002). *A. lwoffii*, which usually does not play a role in hospital-acquired infections, was present in only one specimen. This species seems to be part of the normal skin flora, as it was found in 63 % of the skin specimens of healthy patients (Berlau *et al.*, 1999). In the present work, *A. lwoffii* was isolated from an autologous blood unit, suggesting possible contamination. Three specimens were identified as close to *A. gen. sp. 9*. However, as no comparison is possible with a reference strain and similarity of our isolates with strain CIP 64.7 is below 97 % (Supplementary Table S2) we will not consider this identification as definitive herein. Although *A. gen. sp. 9* and *A. lwoffii* cluster together with high bootstrap support, they are clearly distinguishable by *rpoB* sequence analysis (Fig. 1). The separation of *A. lwoffii* and *A. gen. sp. 9* requires further study, as they are well distinguished by

rpoB, but not by DNA–DNA hybridization, which provisionally remains the gold standard for species delineation (Bouvet & Grimont, 1987; Tjernberg & Ursing, 1989). The identification of 10 % of isolates as *A. haemolyticus* correlated well with a previous report (Seifert *et al.*, 1993). One of the most interesting observations of the present work was the confirmation of *A. schindleri* as a cause of important *Acinetobacter*-related infections. In another study, the majority of the *A. schindleri* strains were isolated from non-sterile body sites of outpatients; the type strain of *A. schindleri* was isolated from urine of a male outpatient with cystitis (Nemec *et al.*, 2001). In contrast, in a recent study, *A. schindleri* was found to represent 4.9 % of non-Acb-complex isolates, with most isolates (80 %) having been recovered from blood (Dortet *et al.*, 2006). As for the identification of species among the Acb complex, the under-reporting of this species is due to problems with its identification (Dortet *et al.*, 2006). However, we could identify our strains as belonging to species with 97.4 % to 100 % sequence similarity with type strain of *A. schindleri* (CIP 107287^T).

In conclusion, our PRBS method is a simple molecular tool for the identification of *Acinetobacter* clinical strains. Allowing rapid and accurate identification of *Acinetobacter* will definitely help in the identification of relationships between emerging *Acinetobacter* species and

Fig. 2. Dendrogram representing phylogenetic relationships between strains of the subset from the Acb complex presented in Table 2. G is seqtype according to Table 2. The tree is derived from the sequences of 16S rRNA gene sequences. MEGA4 software (<http://www.megasoftware.net>) was used to infer the tree using the neighbour-joining method (Kimura two-parameter). GenBank accession numbers with the prefix FJ refer to those 16S rRNA gene sequences obtained in this study. The support of each branch, as determined from 1000 bootstrap samples, is indicated by the value at the node. Bootstrap values below 50 % were omitted. *Acinetobacter ursingii* was used as an outgroup.

clinical outcomes, including strains belonging to species of the Acb complex. It will also be useful in creating a better understanding of the epidemiology of these emerging bacteria.

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