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Acinetobacter ursingii sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens

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The taxonomic status of two recently described phenetically distinctive groups within the genus *Acinetobacter*, designated phenon 1 and phenon 2, was investigated further. The study collection included 51 strains, mainly of clinical origin, from different European countries with properties of either phenon 1 (29 strains) or phenon 2 (22 strains). DNA–DNA hybridization studies and DNA polymorphism analysis by AFLP revealed that these phenons represented two new genomic species. Furthermore, 16S rRNA gene sequence analysis of three representatives of each phenon showed that they formed two distinct lineages within the genus *Acinetobacter*. The two phenons could be distinguished from each other and from all hitherto-described *Acinetobacter* (genomic) species by specific phenotypic features and amplified rDNA restriction analysis patterns. The names *Acinetobacter ursingii* sp. nov. (type strain LUH 3792^T = NIPH 137^T = LMG 19575^T = CNCTC 6735^T) and *Acinetobacter schindleri* sp. nov. (type strain LUH 5832^T = NIPH 1034^T = LMG 19576^T = CNCTC 6736^T) are proposed for phenon 1 and phenon 2, respectively. Clinical and epidemiological data indicate that *A. ursingii* has the capacity to cause bloodstream infections in hospitalized patients.

Keywords: *Acinetobacter ursingii* sp. nov., *Acinetobacter schindleri* sp. nov., polyphasic taxonomy

INTRODUCTION

Over the last 15 years, considerable progress has been made in resolving the taxonomy of the genus *Acinetobacter*. The basis for the present classification was established by Bouvet & Grimont (1986), with the description of 12 DNA–DNA hybridization groups (genomic species) within the genus. This scheme was subsequently extended to include 10 additional genomic species (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989; Gerner-Smidt & Tjernberg, 1993). Seven genomic species have names (*Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter junii*, *Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter radioresistens*), while the others are designated by numbers (reviewed by Janssen *et al*., 1997). Another genomic species ('*Acinetobacter venetianus*') comprising marine oil-degrading organisms was delineated recently (Di Cello *et al*., 1997; Vaneechoutte *et al*., 1999). Nevertheless, the DNA–DNA hybridization studies of Bouvet & Grimont (1986), Tjernberg & Ursing (1989) and Bouvet & Jeanjean (1989) left several strains unclassified, which indicates that the diversity of the genus extends beyond the described groups.

In a recent study, 45 additional unidentifiable isolates were found among 700 clinical isolates from the Czech Republic (Nemec *et al*., 2000). Two groups of isolates (designated phenon 1 and phenon 2) were delineated among the unidentifiable isolates, each of which showed distinctive phenotypic features and amplified rDNA restriction analysis (ARDRA) patterns. The aim of the present study was to define the taxonomic

Abbreviations: ARDRA, amplified rDNA restriction analysis.

The EMBL accession numbers for the 16S rRNA gene sequences of strains LUH 3299, LUH 3792^T, LUH 4763, LUH 4591, LUH 4760 and LUH 5832^T are respectively AJ275037–AJ275041 and AJ278311.

Table 1. Strains of phenon 1 (*Acinetobacter ursingii* sp. nov.) and phenon 2 (*Acinetobacter schindleri* sp. nov.)

All strains were from human specimens. CNCTC, Czech National Collection of Type Cultures, Prague, Czech Republic; LMG, Bacteria Collection, Laboratorium voor Microbiologie Gent, Gent, Belgium; LUH and RUH, Collection L. Dijkshoorn, Leiden University Medical Centre, Leiden, The Netherlands; NIPH, Collection A. Nemec, National Institute of Public Health, Prague, Czech Republic. Abbreviations: CZ, Czech Republic; NL, The Netherlands; NO, Norway; SE, Sweden.

Strain	Other strain designation(s)	Reference/received from	Specimen*	Location and year of isolation
Phenon 1 (*A. ursingii* sp. nov.)				
LUH 3792^T	NIPH 137^T† = LMG 19575^T = CNCTC 6735^T	Nemec *et al.* (2000)	Blood (in)	Praha, CZ, 1993
LUH 4582	NIPH 177†	Nemec *et al.* (2000)	Intravenous line (in)	Praha, CZ, 1993
LUH 4592	NIPH 280†	Nemec *et al.* (2000)	Blood (in)	Sedlčany, CZ, 1994
LUH 3793	NIPH 371†	Nemec *et al.* (2000)	Blood (in)	Příbram, CZ, 1995
LUH 4613	NIPH 375†	Nemec *et al.* (2000)	Pus (in)	Příbram, CZ, 1995
LUH 4614	NIPH 376†	Nemec *et al.* (2000)	Pus (in)	Příbram, CZ, 1995
LUH 4618	NIPH 398†	Nemec *et al.* (2000)	Ulcer (out)	Příbram, CZ, 1996
LUH 4622	NIPH 439†	Nemec *et al.* (2000)	Eye (out)	Sedlčany, CZ, 1996
LUH 5767	NIPH 706†	Nemec *et al.* (2000)	Blood (in)	Příbram, CZ, 1997
LUH 5829	NIPH 950†	Nemec *et al.* (2000)	Blood (in)	Tábor, CZ, 1998
LUH 5830	NIPH 993†	Nemec *et al.* (2000)	Cervix (out)	Příbram, CZ, 1998
LUH 5831	NIPH 1025†	Nemec *et al.* (2000)	Eye (out)	Příbram, CZ, 1998
LUH 5833	NIPH 1048†	Nemec *et al.* (2000)	Blood (in)	Liberec, CZ, 1998
LUH 5834	NIPH 1118†	Nemec *et al.* (2000)	Wound (out)	Sedlčany, CZ, 1999
LUH 5835	NIPH 1120†	Nemec *et al.* (2000)	Urine (out)	Příbram, CZ, 1999
LUH 4761	72a†	Tjernberg & Ursing (1989)	Urine (out)	Malmö, SE, 1980
LUH 4762	93†	Tjernberg & Ursing (1989)	Blood (in)	Malmö, SE, 1980
LUH 4763	119†	Tjernberg & Ursing (1989)	Urine (out)	Malmö, SE, 1980
LUH 4766	166†	Tjernberg & Ursing (1989)	Wound (in)	Malmö, SE, 1981
LUH 4768	175†	Tjernberg & Ursing (1989)	Wound (in)	Malmö, SE, 1981
RUH 1501			Hairy skin (in)	Rotterdam, NL, 1985
RUH 3329	Patient K†	Horrevorts *et al.* (1995)	Blood (in)	Nijmegen, NL, 1990
LUH 3292†		Bernards *et al.* (1997)	Blood (in)	Leiden, NL, 1995
LUH 3299†		Bernards *et al.* (1997)	Blood (in)	Leiden, NL, 1995
LUH 3140		A. T. Bernards	Toes (in)	Enschede, NL, 1995
LUH 3059		A. T. Bernards	Blood (in)	Enschede, NL, 1995
LUH 3324	610/1994‡	J. G. M. Koeleman	Blood	Amsterdam, NL, 1994
LUH 4739	84†	Bouvet & Grimont (1986)	Blood	Unknown
LUH 4828	1614/96‡	D. A. Caugant	Abscess (out)	Kristiansand, NO, 1996
Phenon 2 (*A. schindleri* sp. nov.)				
LUH 5832^T	NIPH 1034^T† = LMG 19576^T = CNCTC 6736^T	Nemec *et al.* (2000)	Urine (out)	Příbram, CZ, 1998
LUH 4590	NIPH 228†	Nemec *et al.* (2000)	Vagina (out)	Praha, CZ, 1994
LUH 4591	NIPH 257†	Nemec *et al.* (2000)	Urine (out)	Hluboká nad Vltavou, CZ, 1993
LUH 4594	NIPH 285†	Nemec *et al.* (2000)	Throat (out)	Příbram, CZ, 1994
LUH 4595	NIPH 286†	Nemec *et al.* (2000)	Ear (out)	Příbram, CZ, 1994
LUH 4597	NIPH 291†	Nemec *et al.* (2000)	Nasal swab (out)	Příbram, CZ, 1994
LUH 4598	NIPH 293†	Nemec *et al.* (2000)	Cervix (out)	Sedlčany, CZ, 1994
LUH 4599	NIPH 296†	Nemec *et al.* (2000)	Cervix (out)	Sedlčany, CZ, 1994
LUH 4612	NIPH 369†	Nemec *et al.* (2000)	Cervix (out)	Sedlčany, CZ, 1994
LUH 4615	NIPH 383†	Nemec *et al.* (2000)	Nasal swab (out)	Milín, CZ, 1996
LUH 5825	NIPH 883†	Nemec *et al.* (2000)	Urine (out)	Příbram, CZ, 1998
LUH 5826	NIPH 900†	Nemec *et al.* (2000)	Conjunctiva (out)	Sedlčany, CZ, 1998
LUH 5827	NIPH 904†	Nemec *et al.* (2000)	Urine (out)	Příbram, CZ, 1998
LUH 5939	NIPH 907†	Nemec *et al.* (2000)	Nasal swab (out)	Příbram, CZ, 1998
LUH 5828	NIPH 933†	Nemec *et al.* (2000)	Vagina (out)	Příbram, CZ, 1998
LUH 4760	60†	Tjernberg & Ursing (1989)	Urine (in)	Malmö, SE, 1980
LUH 4764	120†	Tjernberg & Ursing (1989)	Pleural effusion (in)	Malmö, SE, 1980
LUH 4765	129†	Tjernberg & Ursing (1989)	Urine	Malmö, SE, 1980
RUH 203†		Dijkshoorn *et al.* (1998)	Liquor (out)	Rotterdam, NL, 1983
LUH 4742	594‡	P. J. M. Bouvet	Skin	Unknown
LUH 4743	585‡	P. J. M. Bouvet	Skin	Unknown
LUH 4744	586‡	P. J. M. Bouvet	Skin	Unknown

* If known, specimens from outpatients (out) or inpatients (in) are indicated.

† Strain designation used in a previous publication.

‡ Strain designation as received.

status of these groups by a polyphasic analysis. For this purpose, the collection of Czech strains was enlarged with strains from other European countries that showed characters similar to those of the two phenons.

METHODS

Strains. The 29 strains of phenon 1 and 22 strains of phenon 2 investigated in this study are listed in Table 1. The Czech strains ($n = 30$) were those from the previous study (Nemec *et al.*, 2000). Additionally, 21 strains were selected from a set

Table 2. Biochemical characteristics of phenon 1 (*A. ursingii* sp. nov.) and phenon 2 (*A. schindleri* sp. nov.)

Data are from this study and from Nemec *et al.* (2000). Growth on carbon sources was evaluated after 2 and 6 d of incubation. +, Positive for all strains; −, negative for all strains; numbers are percentages of strains giving a positive reaction. All strains utilized DL-lactate and acetate. None of the strains grew at 44 °C, hydrolysed gelatin, produced haemolysis on sheep-blood agar, acidified Hugh & Leifson's medium with D-glucose or utilized DL-4-aminobutyrate, *β*-alanine, L-histidine, malonate, histamine, L-phenylalanine, phenylacetate, laevulinate, citraconate or L-leucine.

Characteristic	Phenon 1 (*A. ursingii* sp. nov.) ($n = 29$)	Phenon 2 (*A. schindleri* sp. nov.) ($n = 22$)
Growth at 41 °C	−	+*
Growth at 37 °C	+*	+
Utilization of:		
Citrate (Simmons)	+	59
Glutarate	97	95
L-Aspartate	97*	−
Azelate	+	64
D-Malate	+*	95*
4-Hydroxybenzoate	97	64
L-Tartrate	−	18
2,3-Butanediol	−	32
Ethanol	+	95

* Weak growth of some strains.

of about 100 *Acinetobacter* strains isolated by different laboratories that could not be identified as any of the described genomic species. The 21 strains were selected from this set on the basis of phenotypic properties and ARDRA patterns similar to those of the phenon 1 or phenon 2 strains (Nemec *et al.*, 2000). All 51 strains had the properties of the genus *Acinetobacter* (Juni, 1984); i.e. they were Gram-negative, strictly aerobic, oxidase-negative, non-motile coccobacilli and positive in the transformation assay of Juni (1972).

Phenotypic characterization. The tests described by Nemec *et al.* (2000) were used, with the following modifications. Carbon-source utilization tests were supplemented with those for laevulinate, citraconate, 4-hydroxybenzoate, L-tartrate, L-leucine, 2,3-butanediol, ethanol and acetate. The test for *trans*-aconitate utilization was omitted since it may give irreproducible results with some phenon 1 strains. Production of pigments was tested on glycerol-containing media A and B as described by King *et al.* (1954). All tests were performed at 30 °C unless indicated otherwise.

ARDRA. Amplified 16S rDNA was obtained by PCR and analysed by restriction digestion with six restriction endonucleases (*Cfo*I, *Alu*I, *Mbo*I, *Rsa*I, *Msp*I and *Bfa*I) as described previously (Nemec *et al.*, 2000). Interpretation of ARDRA patterns was based on the positions of the fragments of molecular size $\geqslant 100$ bp. The patterns were numbered according to the scheme of Dijkshoorn *et al.* (1998), supplemented by Seifert *et al.* (1997) and Nemec *et al.* (2000).

AFLP fingerprinting. AFLP was performed according to Koeleman *et al.* (1998), with some modifications. DNA was purified as described by Boom *et al.* (1990) and adapters were as described by Vos *et al.* (1995). Restriction and ligation were performed simultaneously at 37 °C for 3 h in a 10 µl volume with 10–50 ng template DNA, 1 U *Eco*RI (Amersham Pharmacia Biotech), 1 U *Mse*I (New England BioLabs), 4 U T4 DNA ligase (Amersham Pharmacia Biotech), 1 × T4 DNA ligase buffer, 500 ng BSA, 50 mM NaCl, 2 pmol *Eco*RI adapters and 20 pmol *Mse*I adapters. After incubation, the mixture was diluted with 10 mM Tris/HCl, 0·1 mM EDTA (pH 8·0) to a final volume of 200 µl. Five microlitres diluted mixture was added to a final volume of 10 µl reaction mixture containing 20 ng Cy5-labelled *Eco*RI + A primer (Cy5-GACTGCGTACCAA-TTCa-3′; where a is a selective A base), 60 ng *Mse*I + C primer (5′-GATGAGTCCTGAGTAAc-3′; where c is a selective C base), 1 × *Taq* polymerase buffer, 1·5 mM $MgCl_2$, 0·2 mM (each) dNTP and 1 U Goldstar *Taq* DNA polymerase (Eurogentec). Amplification with a Progene thermocycler (Techne) was as follows: 2 min at 72 °C and 2 min at 94 °C; one cycle of 30 s at 94 °C, 30 s at 65 °C and 60 s at 72 °C; 12 cycles of 30 s at 94 °C, 30 s at a temperature of 0·7 °C lower than the previous cycle, starting at 64·3 °C, followed by 60 s at 72 °C; 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C; and a final cycle of 10 min at 72 °C. PCR products were mixed with 3 µl formamide containing 0·5 % dextran blue, heated for 5 min at 95 °C and cooled on ice. Samples of 3 µl were loaded on a denaturing polyacrylamide gel (ReproGel High Resolution; Amersham Pharmacia Biotech) with 200 mm standard thermoplates. Fragment separation was performed using the ALFexpress II DNA analysis system (Amersham Pharmacia Biotech) for 500 min at 55 °C and 30 W constant power with 2 s sampling intervals. The peak patterns generated were converted to TIF files, which were analysed by the BioNumerics 2.0 software package (Applied Maths). Fragments in the range 50–500 bp were used for cluster analysis. Pearson's product-moment coefficient (r) was used as a measure of similarity and grouping was obtained by the unweighted pair group average linked method (UPGMA).

DNA–DNA hybridization. The two-step elution procedure was used to determine DNA–DNA relatedness (Tjernberg *et al.*, 1989). By this method, ^{125}I-labelled DNA probes from strains LUH 3792^T (phenon 1) and LUH 5832^T (phenon 2) were hybridized on a filter with unlabelled DNAs of the

Table 3. ARDRA patterns of phenon 1 and phenon 2 strains

Data were from this study and from Nemec *et al.* (2000). Pattern designation according to Dijkshoorn *et al.* (1998) and Nemec *et al.* (2000). ND, Not determined; New, novel patterns.

Strain(s)	Restriction pattern with:					
	*Cfo*I	*Alu*I	*Mbo*I	*Rsa*I	*Msp*I	*Bfa*I
Phenon 1 (*A. ursingii* sp. nov.)						
LUH 3792^T, LUH 3292, LUH 3299, LUH 4592, LUH 4614, LUH 4622, LUH 4762, LUH 4763, LUH 4766, LUH 4828, LUH 5829, LUH 5830, LUH 5834, RUH 1501, RUH 3329	1	4	3	5	3	ND
LUH 3059, LUH 3793, LUH 4582, LUH 4768	1	4+nw*	3	4	3	ND
LUH 3140, LUH 5767, LUH 5835	1	4+nw*	3	4+5	3	ND
LUH 3324, LUH 4761	1	4+nw*	1+3	4+5	3	ND
LUH 4739	1	4	1+3	5	3	ND
LUH 4618	1	4	3	4+5	3	ND
LUH 5833	1	4	1+3	4+5	3	ND
LUH 5831	1	4	3	2+5	3	ND
LUH 4613	1	4†	1+3	2+5	3	ND
Phenon 2 (*A. schindleri* sp. nov.)						
LUH 5832^T, LUH 4591, LUH 4595, LUH 4597, LUH 4598, LUH 4599, LUH 4615, LUH 4742, LUH 4760, LUH 4764, LUH 5825, LUH 5827, LUH 5828, LUH 5939, RUH 203	1+5	2+4‡	1	2	2	10
LUH 4590, LUH 4594, LUH 4612, LUH 4743, LUH 4744, LUH 5826	1+5	2	1	2	2	10
LUH 4765	5	New	1	2	2	New

* A combined *Alu*I pattern, tentatively interpreted as the mixture of pattern 4 and a new pattern (Nemec *et al.*, 2000; Fig. 1).

† Pattern 4 containing an additional, weak band of approximately 223 bp; this pattern is highly similar to combined *Alu*I pattern 2+4 (Nemec *et al.*, 2000).

‡ The band (220 bp) specific for *Alu*I pattern 2 was diffuse in all strains (Nemec *et al.*, 2000; Fig. 1).

phenon 1 and phenon 2 strains and reference strains of all described *Acinetobacter* genomic species. The amount of DNA released from the filter was measured at two temperatures, at 7 °C below the thermal melting midpoint of the homologous duplex and at 100 °C. The amount of DNA released in the first step expressed as a percentage of the total amount of eluted DNA at 100 °C (%DR7) was the criterion for inclusion of strains in a species, with the intraspecies and interspecies values for %DR7 being $\leqslant 26$ and $\geqslant 37$, respectively (Tjernberg *et al.*, 1989). Each %DR7 value was calculated as a mean of at least two hybridization experiments.

16S rDNA sequencing and comparative analysis. A fragment of the 16S rRNA gene (corresponding to positions 10–1507 in the *Escherichia coli* numbering system) of three phenon 1 strains (LUH 3792^T, LUH 3299, LUH 4763) and three phenon 2 strains (LUH 5832^T, LUH 4591, LUH 4760) was sequenced as described by Vaneechoutte *et al.* (2000). The 16S rDNA sequences obtained for phenon 1 and phenon 2 strains were compared with the sequences representing all described *Acinetobacter* genomic species, i.e. 21 sequences determined by Ibrahim *et al.* (1997) (EMBL accession numbers Z93434–Z93454) and the sequence of '*A. venetianus*' strain RAG-1 (AJ295007), and the sequences of *Moraxella lacunata* ATCC 17967^T (AF005160) and *Psychrobacter immobilis* ATCC 43116^T (U39399). All steps of the comparative sequence analysis were performed by using the GeneBase software package (Applied Maths). Firstly, pairwise alignment using UPGMA was carried out with a gap penalty of 100%, a unit gap cost of 20% and an ambiguity cost of 50% of the mismatch cost. Subsequently, global alignment with *P. immobilis* as the outgroup was carried out on the region corresponding to positions 67–1444 of the 16S rRNA gene of *E. coli*, with costs as above. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20%. The neighbour-joining method was used to construct the dendrogram based on this similarity matrix.

RESULTS AND DISCUSSION

Phenotypic characteristics

Colonies of all strains grown on nutrient agar after 24 h were circular, convex, smooth and slightly opaque with entire margins. The colonies of phenon 1 strains

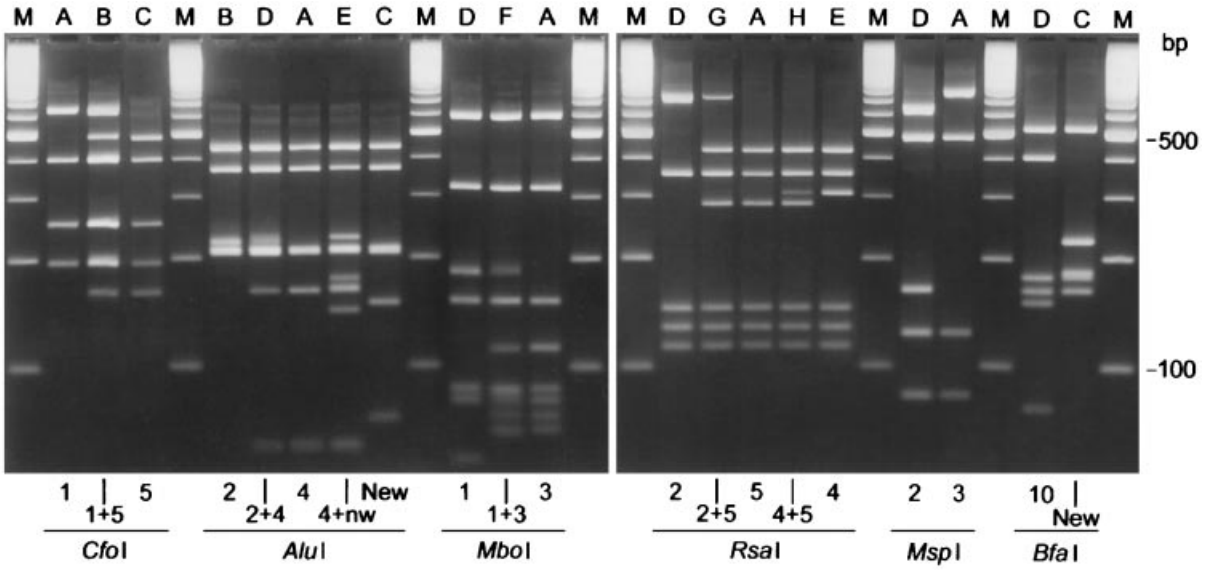

Fig. 1. Overview of the ARDRA patterns found in phenon 1 (*A. ursingii* sp. nov.) and phenon 2 (*A. schindleri* sp. nov.) strains. Strains are indicated by upper-case letters above the lanes: A, LUH 3792^T (phenon 1); B, LUH 4594 (phenon 2); C, LUH 4765 (phenon 2); D, LUH 5832^T (phenon 2); E, LUH 3793 (phenon 1); F, LUH 4761 (phenon 1); G, LUH 4613 (phenon 1); H, LUH 4618 (phenon 1). Lanes M, molecular size markers (100-bp ladder). Pattern designations for the various enzymes are given below the lanes.

were respectively 1–1·5 mm and 1·5–3 mm in diameter after 24 h and 48 h of incubation. The colonies of phenon 2 strains were respectively 1·5–2·5 and 2–4·5 mm in diameter after 24 h and 48 h of incubation. Some phenon 2 strains (e.g. LUH 5832^T, LUH 4615 and LUH 4764) produced diffuse, light yellowish-brown pigment on King's medium A and were surrounded by dark greenish zones on sheep-blood agar.

Biochemical test results are given in Table 2. The strains of phenon 1 were, with few exceptions, biochemically uniform, while those of phenon 2 varied in the utilization of citrate (Simmons), azelate, 4-hydroxybenzoate, L-tartrate and 2,3-butanediol. Growth of some strains of both phenons on D-malate and of some phenon 1 strains on L-aspartate was weak after 6 d and became more apparent after prolonged incubation (up to 10 d).

ARDRA

ARDRA patterns of the phenon 1 and phenon 2 strains are summarized in Table 3 and Fig. 1. Most phenon 1 strains shared the recently described *Rsa*I pattern 5 (Nemec *et al.*, 2000), which differs slightly from pattern 4 in migration of a fragment of about 300 bp (Fig. 1). Based on the analysis of the 16S rDNA sequences in the present study, this difference can be explained by the presence of an additional *Rsa*I restriction site responsible for a 22 bp truncation of the fragment in *Rsa*I pattern 5. Accordingly, the previously published *Rsa*I patterns 4 of strains LUH 3292, LUH 3299 and LUH 3329 (Bernards *et al.*, 1997; Dijkshoorn *et al.*, 1998) were reinterpreted as *Rsa*I patterns 5. Some of the ARDRA patterns appeared to be mixtures of two known single patterns. Of these, *Alu*I 2+4 and *Cfo*I 1+5 patterns were found in most phenon 2 strains. However, the band specific for *Alu*I pattern 4 (162 kb) was very weak in some of these strains (e.g. RUH 203 and LUH 4760) and could only be seen clearly when the gel was overloaded with DNA. Similarly, RUH 203 and LUH 4590 yielded very faint bands of 160 and 479 kb specific for *Cfo*I pattern 5. This observation may explain the difference between the published *Cfo*I pattern 1 and *Alu*I pattern 2 of strain RUH 203 (Dijkshoorn *et al.*, 1998) and those of the present study.

AFLP fingerprinting

Reproducibility of AFLP as determined by testing several control strains was always higher than 90% (data not shown). Cluster analysis of the phenon 1 and 2 strains was performed together with a total of 200 strains from all described *Acinetobacter* genomic species (identified by DNA–DNA hybridization). The strains of each of the described genomic species formed a separate cluster at a cut-off level of about 50% (data not shown). Clustering of all phenon 1 and phenon 2 strains and one representative strain of each described *Acinetobacter* genomic species is shown in Fig. 2. The strains of phenon 1 and phenon 2 grouped in two clusters at levels of 67 and 63% and were clearly separated from each other, and from all other strains at 33 and 20%, respectively.

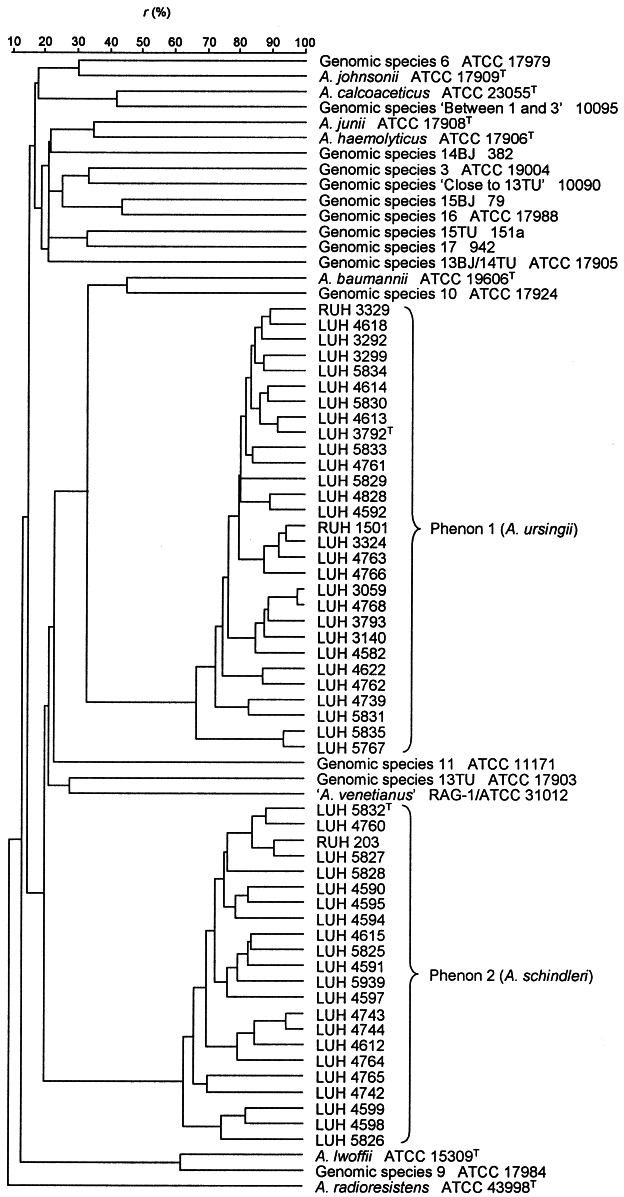

Fig. 2. UPGMA/product-moment cluster analysis of the AFLP fingerprints of 29 strains of phenon 1 (*A. ursingii* sp. nov.), 22 strains of phenon 2 (*A. schindleri* sp. nov.) and 22 strains representing all hitherto-described (genomic) species of the genus *Acinetobacter*. The latter strains are designated by either the ATCC numbers or the numbers used in previous DNA–DNA hybridization studies (Tjernberg & Ursing, 1989; Bouvet & Jeanjean, 1989; Gerner-Smidt & Tjernberg, 1993). Levels of correlation are expressed as percentages of similarity for convenience.

DNA–DNA hybridization

The %DR7 values obtained with radiolabelled DNA from strains LUH 3792^T and LUH 5832^T are summarized in Table 4. The intraphenon range of the %DR7 values corresponded to the intraspecies variability of %DR7 values found previously (Tjernberg *et al.*, 1989), the only exception being LUH 4590 (phenon 2), with %DR7 = 30. However, although the latter value was relatively high, it was significantly lower than the values found for hybridization with both the reference strains of the described genomic species and phenon 1 strains. Thus, the %DR7 values support the conclusion that the strains of phenon 1 and phenon 2 represent novel, distinctive genomic groups.

16S rDNA sequence analysis

The 16S rDNA sequences of phenon 1 strains LUH 3792^T, LUH 3299 and LUH 4763 were identical, and the sequences of phenon 2 strains LUH 5832^T, LUH 4591 and LUH 4760 were nearly identical (99·4% similarity). A dendrogram based on the comparison of these sequences with those representing known *Acinetobacter* genomic species and the closest genera is shown in Fig. 3. Both phenon 1 and phenon 2 strains clustered with the other members of the genus *Acinetobacter* and were well separated from their neighbours. The similarity values between the 16S rDNA sequence of the phenon 1 strains and those of the other members of the genus *Acinetobacter* were in the range 95·4–97·3%; the similarity between the sequences of the phenon 2 strains and those of the other members of the genus ranged from 95·4 to 98·0%. The lowest intrageneric 16S rDNA sequence similarity (95·4%) was observed between phenon 1 and phenon 2 strain LUH 4760.

Taxonomic status of phenon 1 and phenon 2

The results of DNA–DNA hybridization and AFLP confirmed that phenon 1 and phenon 2 represent two distinctive genomic species, different from all hitherto-described *Acinetobacter* genomic species. Furthermore, comparative analysis of 16S rDNA sequences indicated that phenon 1 and phenon 2 formed two distinct lineages within the genus *Acinetobacter*. Both phenons could be differentiated from the other genomic species of the genus and from each other by ARDRA patterns and biochemical characters (see below). On the basis of these findings, phenon 1 and phenon 2 described by Nemec *et al.* (2000) represent two novel species of the genus *Acinetobacter*, for which the respective names *Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov. are proposed.

Differentiation and identification

The array of 19 biochemical tests suggested by Bouvet & Grimont (1987) allowed unambiguous identification of almost all strains of *A. ursingii* and *A. schindleri*. Comparison of our results with those of previous studies (Bouvet & Grimont, 1987; Gerner-Smidt *et al.*, 1991; Vaneechoutte *et al.*, 1999) showed that both novel species could be differentiated from most other genomic species of the genus *Acinetobacter* by their inability to grow at 44 °C, to oxidize D-glucose, to hydrolyse gelatin and to utilize DL-4-aminobutyrate, *β*-alanine, L-histidine, malonate, histamine, L-phenylalanine and phenylacetate. Growth at 41 and 37 °C

Table 4. Results of DNA–DNA hybridization using the %DR7 coefficient

The reference strains were those used in the studies of Tjernberg & Ursing (1989) and Bouvet & Jeanjean (1989), strains 10095 and 10090 (Gerner-Smidt & Tjernberg, 1993) and '*A. venetianus*' strains C3 and RAG-1 (Di Cello *et al.*, 1997; Vaneechoutte *et al.*, 1999). Values are means ± SD, with the range in parentheses.

Source of unlabelled DNA	Labelled DNA from LUH 3792^T (phenon 1, *A. ursingii* sp. nov.)	Labelled DNA from LUH 5832^T (phenon 2, *A. schindleri* sp. nov.)
Phenon 1 strains ($n = 29$)	12 ± 4 (7–25)	–
Reference strains of the known genomic species ($n = 22$) and phenon 2 strains LUH 4590 and LUH 4765	46 ± 5 (37–54)	–
Phenon 2 strains ($n = 22$)	–	22 ± 4 (14–30)
Reference strains of the known genomic species ($n = 22$) and phenon 1 strains LUH 3792^T, LUH 4761 and LUH 4828	–	57 ± 4 (49–70)

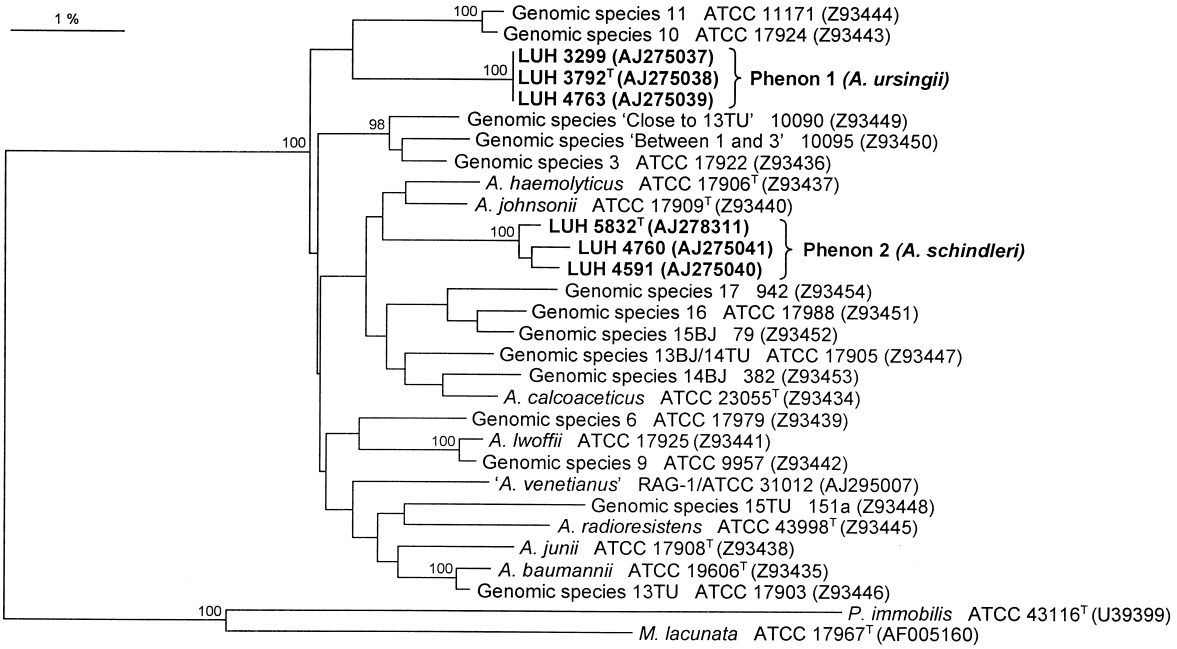

Fig. 3. Rooted 16S rDNA sequence-based tree showing the relationship of phenon 1 (*A. ursingii* sp. nov.), phenon 2 (*A. schindleri* sp. nov.), the other members of the genus *Acinetobacter*, *Moraxella lacunata* and *Psychrobacter immobilis* (the outgroup). The tree was constructed using the neighbour-joining method. The numbers at the branching points are the proportions of 100 bootstrap resamplings that support the tree topology (only values above 90% are shown). EMBL accession numbers are given in parentheses. Bar, 1% estimated sequence divergence.

and utilization of glutarate and L-aspartate were the most useful tests for differentiating *A. ursingii* and *A. schindleri* from each other and from *A. junii*, *A. johnsonii*, *A. lwoffii* and genomic species 15TU (Table 5). Only two strains could not be identified unambiguously; *A. ursingii* LUH 4614 failed to grow on L-aspartate and therefore could not be differentiated from *A. schindleri*, while *A. schindleri* LUH 5939 did not utilize glutarate and consequently could not be distinguished from genomic species 15TU and *A. lwoffii*. However, LUH 5939 could be differentiated from *A. lwoffii* by its ability to utilize 4-hydroxybenzoate, which is not included in the identification scheme of Bouvet & Grimont (1987).

None of the ARDRA profiles of the *A. ursingii* and *A. schindleri* strains have been observed previously in any of the known genomic species (Dijkshoorn *et al.*, 1998; Seifert *et al.*, 1997; Vaneechoutte *et al.*, 1999). All but one of the *A. schindleri* strains yielded *Bfa*I pattern 10, which may be particularly useful in their differentiation from *A. johnsonii* strains that have highly similar pattern combinations with the other enzymes used in ARDRA (Seifert *et al.*, 1997). A total of nine different

Table 5. Phenotypic characters useful for discrimination of *A. ursingii* and *A. schindleri* and for their differentiation from phenotypically similar (genomic) species

Data for *A. junii*, *A. johnsonii*, *A. lwoffii* and genomic species 15TU were taken from Gerner-Smidt *et al.* (1991). +, Positive for 90–100% of strains; −, positive for 0–10% of strains; D, positive for 11–89% of strains.

Characteristic	*A. ursingii*	*A. schindleri*	*A. junii*	*A. johnsonii*	*A. lwoffii*	Genomic species 15TU
Growth at 41 °C	−	+	D	−	−	D
Growth at 37 °C	+	+	+	−	D	+
Utilization of:						
Glutarate	+	+	−	−	−	−
L-Aspartate	+	−	−	D	−	−

ARDRA profiles were encountered among the *A. ursingii* strains. In spite of this variability, several pattern combinations may be useful for the identification of *A. ursingii*, e.g. the combination of *Cfo*I 1, *Mbo*I 3 or *Mbo*I 1+3 and *Msp*I 3 or the combination of *Rsa*I 4 or *Rsa*I 5 or *Rsa*I 4+5 or *Rsa*I 2+5 and *Msp*I 3.

Clinical importance

The available clinical and epidemiological data suggest that *A. ursingii* and *A. schindleri* differ in their distribution in patients. While the majority of the *A. schindleri* strains were isolated from non-sterile body sites of outpatients, *A. ursingii* comprised mainly clinically significant isolates from seriously ill hospitalized patients. Almost half of the *A. ursingii* strains were isolated from blood cultures and at least some of them were recovered from patients with diagnosed bacteraemia or septicaemia (Bernards *et al.*, 1997; Horrevorts *et al.*, 1995; Nemec *et al.*, 2000). Moreover, the identity of typing characters that was found in two epidemiologically related isolates (Nemec *et al.*, 2000) indicates that *A. ursingii* strains have the potential to spread among patients.

Description of *Acinetobacter ursingii* sp. nov.

Acinetobacter ursingii (ur.sin′gi.i. N.L. gen. masc. n. *ursingii* in honour of Jan Ursing, the recently deceased Swedish bacteriologist and taxonomist).

Characteristics correspond to those of the genus (Juni, 1984). Colonies on nutrient agar after 24 h incubation at 30 °C are approximately 1·0–1·5 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs at 37 °C but not at 41 °C. Acid is not produced from D-glucose, sheep blood is not haemolysed and gelatin is not hydrolysed. DL-Lactate, citrate (Simmons), azelate, D-malate, ethanol and acetate are utilized as sole sources of carbon and energy. Glutarate, L-aspartate and 4-hydroxybenzoate are utilized by most strains. DL-4-Aminobutyrate, β-alanine, L-histidine, malonate, histamine, L-phenylalanine, phenylacetate, laevulinate, citraconate, L-tartrate, L-leucine and 2,3-butanediol are not utilized.

The type strain is LUH 3792^T (= NIPH 137^T = LMG 19575^T = CNCTC 6735^T), isolated from blood of a hospitalized patient with endocarditis. This strain utilizes glutarate, L-aspartate and 4-hydroxybenzoate. The restriction patterns of amplified 16S rDNA of the type strain are *Cfo*I 1, *Alu*I 4, *Mbo*I 3, *Rsa*I 5, *Msp*I 3. The EMBL accession number for its 16S rDNA sequence is AJ275038.

Description of *Acinetobacter schindleri* sp. nov.

Acinetobacter schindleri (schin′dle.ri. N.L. gen. masc. n. *schindleri* in honour of Jiří Schindler, Czech microbiologist and taxonomist).

Characteristics correspond to those of the genus (Juni, 1984). Colonies on nutrient agar after 24 h incubation at 30 °C are approximately 1·5–2·5 mm in diameter, circular, convex, smooth and slightly opaque with entire margins. Growth occurs at 41 °C but not at 44 °C. Acid is not produced from D-glucose, sheep blood is not haemolysed and gelatin is not hydrolysed. DL-Lactate and acetate are utilized as sole sources of carbon and energy. Glutarate, D-malate and ethanol are utilized by most strains. Various numbers of strains utilize citrate (Simmons), azelate, 4-hydroxybenzoate, L-tartrate and 2,3-butanediol. DL-4-Aminobutyrate, L-aspartate, β-alanine, L-histidine, malonate, histamine, L-phenylalanine, phenylacetate, laevulinate, citraconate and L-leucine are not utilized.

The type strain is LUH 5832^T (= NIPH 1034^T = LMG 19576^T = CNCTC 6736^T), isolated from urine of a male outpatient with cystitis. This strain utilizes citrate (Simmons), glutarate, D-malate, 4-hydroxybenzoate and ethanol but not azelate, L-tartrate or 2,4-butanediol. The restriction patterns of the amplified 16S rDNA of the type strain are *Cfo*I 1+5, *Alu*I 2+4, *Mbo*I 1, *Rsa*I 2, *Msp*I 2, *Bfa*I 10. The EMBL accession number for its 16S rDNA sequence is AJ278311.

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REFERENCES

Bernards, A. T., de Beaufort, A. J., Dijkshoorn, L. & van Boven, C. P. A. (1997). Outbreak of septicaemia in neonates caused by *Acinetobacter junii* investigated by amplified ribosomal DNA restriction analysis (ARDRA) and four typing methods. *J Hosp Infect* **35**, 129–140.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim-van Dillen, P. M. E. & van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *J Clin Microbiol* **28**, 495–503.

Bouvet, P. J. M. & Grimont, P. A. D. (1986). Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Bacteriol* **36**, 228–240.

Bouvet, P. J. M. & Grimont, P. A. D. (1987). Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann Inst Pasteur Microbiol* **138**, 569–578.

Bouvet, P. J. M. & Jeanjean, S. (1989). Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Res Microbiol* **140**, 291–299.

Di Cello, F., Pepi, M., Baldi, F. & Fani, R. (1997). Molecular characterization of an *n*-alkane-degrading bacterial community and identification of a new species, *Acinetobacter venetianus*. *Res Microbiol* **148**, 237–249.

Dijkshoorn, L., van Harsselaar, B., Tjernberg, I., Bouvet, P. J. M. & Vaneechoutte, M. (1998). Evaluation of amplified ribosomal DNA restriction analysis for identification of *Acinetobacter* genomic species. *Syst Appl Microbiol* **21**, 33–39.

Gerner-Smidt, P. & Tjernberg, I. (1993). *Acinetobacter* in Denmark: II. Molecular studies of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex. *APMIS* **101**, 826–832.

Gerner-Smidt, P., Tjernberg, I. & Ursing, J. (1991). Reliability of phenotypic tests for identification of *Acinetobacter* species. *J Clin Microbiol* **29**, 277–282.

Horrevorts, A., Bergman, K., Kollée, L., Breuker, I., Tjernberg, I. & Dijkshoorn, L. (1995). Clinical and epidemiological investigations of *Acinetobacter* genomospecies 3 in a neonatal intensive care unit. *J Clin Microbiol* **33**, 1567–1572.

Ibrahim, A., Gerner-Smidt, P. & Liesack, W. (1997). Phylogenetic relationship of the twenty-one DNA groups of the genus *Acinetobacter* as revealed by 16S ribosomal DNA sequence analysis. *Int J Syst Bacteriol* **47**, 837–841.

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K. & Dijkshoorn, L. (1997). Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting. *Int J Syst Bacteriol* **47**, 1179–1187.

Juni, E. (1972). Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. *J Bacteriol* **112**, 917–931.

Juni, E. (1984). Genus III. *Acinetobacter* Brisou and Prévot 1954, 727^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 303–307. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

King, E. O., Ward, W. K. & Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescein. *J Lab Clin Med* **44**, 301–307.

Koeleman, J. G. M., Stoof, J., Biesmans, D. J., Savelkoul, P. H. & Vandenbroucke-Grauls, C. M. J. E. (1998). Comparison of amplified ribosomal DNA restriction analysis, random amplified polymorphic DNA analysis, and amplified fragment length polymorphism fingerprinting for identification of *Acinetobacter* genomic species and typing of *Acinetobacter baumannii*. *J Clin Microbiol* **36**, 2522–2529.

Nemec, A., Dijkshoorn, L. & Ježek, P. (2000). Recognition of two novel phenons of the genus *Acinetobacter* among non-glucose-acidifying isolates from human specimens. *J Clin Microbiol* **38**, 3937–3941.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I. & Vaneechoutte, M. (1997). Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *J Clin Microbiol* **35**, 2819–2825.

Tjernberg, I. & Ursing, J. (1989). Clinical strains of *Acinetobacter* classified by DNA–DNA hybridization. *APMIS* **97**, 595–605.

Tjernberg, I., Lindh, E. & Ursing, J. (1989). A quantitative bacterial dot method for DNA–DNA hybridization and its correlation to the hydroxyapatite method. *Curr Microbiol* **18**, 77–81.

Vaneechoutte, M., Tjernberg, I., Baldi, F., Pepi, M., Fani, R., Sullivan, E. R., van der Toorn, J. & Dijkshoorn, L. (1999). Oil-degrading *Acinetobacter* strain RAG-1 and strains described as '*Acinetobacter venetianus* sp. nov.' belong to the same genomic species. *Res Microbiol* **150**, 69–73.

Vaneechoutte, M., Claeys, G., Steyaert, S., De Baere, T., Peleman, R. & Verschraegen, G. (2000). Isolation of *Moraxella canis* from an ulcerated metastatic lymph node. *J Clin Microbiol* **38**, 3870–3871.

Vos, P., Hogers, R., Bleeker, M. & 8 other authors (1995). AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res* **23**, 4407–4414.