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Seven novel species of *Acinetobacter* isolated from activated sludge

Emma L. Carr,[1] Peter Kämpfer,[2] Bharat K. C. Patel,[3] Volker Gürtler[4] and Robert J. Seviour[1]

Correspondence
Emma L. Carr
emma.carr@bendigo.latrobe.edu.au

[1]Biotechnology Research Centre, La Trobe University, Bendigo, Victoria 3552, Australia

[2]Institut für Angewandte Mikrobiologie, Justus-Liebig-Universität, Giessen D35390, Germany

[3]Faculty of Science and Technology, Griffith University, Nathan, Brisbane, Queensland 4111, Australia

[4]Microbiology Dept, Austin and Repatriation Hospital, Melbourne, Victoria, Australia

Thirteen isolates of *Acinetobacter* were obtained from activated sludge plants in Victoria, Australia. Earlier 16S–23S rDNA genomic fingerprinting and partial 16S rDNA sequence data had suggested that these isolates might contain previously undescribed species. This view was confirmed here. A polyphasic taxonomic approach involving phenotypic characterization, near-complete 16S rDNA sequence data and DNA–DNA hybridization analyses support the view that seven novel genomic species can be differentiated in this group of isolates. However, when fluorescence *in situ* hybridization (FISH) studies were performed with a 16S-rRNA-targeted probe specific for the genus *Acinetobacter*, used to identify *Acinetobacter* in activated sludge plants, all these strains responded positively. This suggests that these isolates would not have been missed in earlier FISH studies where their role as polyphosphate-accumulating bacteria has been questioned. This report describes these isolates and proposes that they be named *Acinetobacter baylyi* (type strain B2^T = DSM 14961^T = CIP 107474^T), *Acinetobacter bouvetii* (type strain 4B02^T = DSM 14964^T = CIP 107468^T), *Acinetobacter grimontii* (type strain 17A04^T = DSM 14968^T = CIP 107470^T), *Acinetobacter tjernbergiae* (type strain 7N16^T = DSM 14971^T = CIP 107465^T), *Acinetobacter towneri* (type strain AB1110^T = DSM 14962^T = CIP 107472^T), *Acinetobacter tandoii* (type strain 4N13^T = DSM 14670^T = CIP 107469^T) and *Acinetobacter gerneri* (type strain 9A01^T = DSM 14967^T = CIP 107464^T).

INTRODUCTION

Because of their ubiquitous nature and clinical importance, members of the genus *Acinetobacter* continue to attract interest. Currently, isolates can be confidently and unequivocally assigned to the genus *Acinetobacter* using the transformation assay of Juni (1972). 16S rDNA sequence analysis has also shown that *Acinetobacter* spp. represent a well-defined genus (Ibrahim *et al.*, 1997). However, species delineation has been more problematic and although a total of 24 genomic species have so far been recognized, only nine of these have been provided with valid species names (Bouvet & Grimont, 1986; Nemec *et al.*, 2001). Bouvet & Grimont (1986) described the first 12 genomic species of *Acinetobacter*, including six that were given valid names, i.e. *Acinetobacter calcoaceticus*, *Acinetobacter baumannii*, *Acinetobacter haemolyticus*, *Acinetobacter junii*, *Acinetobacter johnsonii* and *Acinetobacter lwoffii*. Five additional proteolytic genomic species were subsequently delineated (Bouvet & Jeanjean, 1989), but none of these were named. Tjernberg & Ursing (1989) described three more genomic species among their clinical isolates and their DNA group 14 was shown to be identical to genomic species 13 of Bouvet & Jeanjean (1989). Gerner-Smidt & Tjernberg (1993) found two additional genomic species that they showed were closely related, but not identical, to the *A. calcoaceticus–A. baumannii* complex (*Acb* complex). Subsequent descriptions of two species, *Acinetobacter ursingii* and *Acinetobacter schindleri*, also from clinical sources, were published by Nemec *et al.* (2001).

While the majority of strains of described species have been isolated from clinical sources, many of the described species also include environmental strains. Overall, the ecology of species belonging to the genus *Acinetobacter* is not well

Abbreviations: FISH, fluorescence *in situ* hybridization; pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl; vsP, variance of separation potential.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains B2^T, AB1110^T, 7N16^T, 4B02^T, 17A04^T, 9A01^T and 4N13^T are AF509820, AF509823, AF509825, AF509827, AF509828, AF509829 and AF509830, respectively.

elucidated. *Acinetobacter radioresistens* isolated from cotton (Nishimura *et al.*, 1988) and an oil-degrading *Acinetobacter* [invalidly named '*Acinetobacter venetianus*' (Di Cello *et al.*, 1997)] represent two of the genomic species of *Acinetobacter* isolated from environmental sources. Notable among the habitats occupied by *Acinetobacter* species is activated sludge (Fuhs & Chen, 1975; Buchan, 1983; Cloete & Steyn, 1987; Beacham *et al.*, 1990; Knight *et al.*, 1993). Since *Acinetobacter* spp. were once thought to be responsible for the biological removal of phosphate from wastewater, work has been done looking at isolates from this environment. Molecular probing using fluorescence *in situ* hybridization (FISH) has suggested that *Acinetobacter* is not a significant or important phosphate-accumulating bacterial population as only a small percentage could be detected in these systems by FISH with the genus-specific probes described by Wagner *et al.* (1994) and Snaidr *et al.* (1997). Furthermore, clone library studies have failed to demonstrate their presence in large numbers in activated sludge systems actively removing phosphorus (Bond *et al.*, 1995, 1999).

Several studies have described strains that could not be identified as known genomic species of *Acinetobacter*. Even among the clinical isolates characterized to date, some strains in many of the studies have not been assigned to any of the known genomic species (Tjernberg & Ursing, 1989; Bouvet & Grimont; 1986; Nishimura *et al.*, 1988; Gerner-Smidt & Tjernberg, 1993). The few studies with *Acinetobacter* isolates from activated sludge have shown repeatedly that many of these do not fit into the already described DNA groups (Maszenan *et al.*, 1997; Carr *et al.*, 2001a, b) and Soddell *et al.* (1993) concluded that none of the phenotypic identification schemes designed for clinical isolates of *Acinetobacter* were suitable for their identification. These findings imply that the genus *Acinetobacter* is much more diverse taxonomically than data from clinical isolates would suggest; this proposal receives support from the work of Carr *et al.* (2001b), who fingerprinted the 16S–23S rDNA spacer region of *Acinetobacter* isolates from activated sludge systems in Australia. Results showed that few of these environmental strains grouped closely with the known genomic species. Based on these genomic fingerprints and partial (first 500 bp) 16S rDNA sequences (E. Carr, unpublished data), several of these *Acinetobacter* strains were selected for further taxonomic study. Phenotypic characteristics and 16S rDNA sequence and DNA–DNA hybridization data support the view that these strains represent seven novel genomic species of *Acinetobacter*: *Acinetobacter baylyi*, *Acinetobacter grimontii*, *Acinetobacter tjernbergiae*, *Acinetobacter bouvetii*, *Acinetobacter towneri*, *Acinetobacter tandoii* and *Acinetobacter gerneri*.

METHODS

Strains. A total of 13 *Acinetobacter* strains from activated sludge plants in Victoria, Australia, was selected for systematic studies on the basis of genomic fingerprinting of their 16S–23S rDNA intergenic spacer regions (Carr *et al.*, 2001b). These strains, their sites of isolation and culture collection numbers are given in Table 1. All could be assigned to the genus *Acinetobacter* by the transformation assay of Juni (1972). The known genomic species used in this study were the type strains described by Bouvet & Grimont (1986) (denoted BG), Bouvet & Jeanjean (1989) (denoted BJ), Tjernberg & Ursing (1989) (denoted TU) and Nemec *et al.* (2001), which are held at Giessen University by P. Kämpfer. The type strains of the recently described species *A. ursingii* and *A. schindleri* were kindly obtained from M. Vaneechoutte, Belgium. In addition to these activated sludge strains, 198 *Acinetobacter* strains listed by Gerner-Smidt *et al.* (1991) were included in the phenotypic section of the work. All these have been previously assigned by them to genomic species of *Acinetobacter* by DNA–DNA hybridization.

Culture conditions and DNA isolation. All cultures were grown on R2A medium (Reasoner & Geldreich, 1985) at 30 °C for 48 h. Chromosomal DNA was extracted from overnight cultures using the Promega Wizard Genomic DNA Purification kit, according to the manufacturer's instructions, resuspended in distilled water, run on an agarose gel to check integrity and then stored at −20 °C until used.

Table 1. Details of activated sludge *Acinetobacter* strains used in this study

Acinetobacter strain no.	Biolog identification	Origin of isolation	Culture collection no.	GenBank acc. no.	Proposed species name
4B02^T	UN	Bendigo, Australia	DSM 14964^T (=CIP 107468^T)	AF509827	*Acinetobacter bouvetii*
B2^T	UN	Bendigo, Australia	DSM 14961^T (=CIP 107474^T)	AF509820	*Acinetobacter baylyi*
A7	UN	Albury, Australia	DSM 14959 (=CIP 107476)	AF509822	*Acinetobacter baylyi*
C5	UN	Ballarat, Australia	DSM 14963 (=CIP 107473)	AF509821	*Acinetobacter baylyi*
AB1110^T	BG7	Bendigo, Australia	DSM 14962^T (=CIP 107472^T)	AF509823	*Acinetobacter towneri*
A23	UN	Albury, Australia	DSM 14960 (=CIP 107475)	AF509832	
4N13^T	UN	Bendigo, Australia	DSM 14670^T (=CIP 107469^T)	AF509830	*Acinetobacter tandoii*
2N01	BG12	Bendigo, Australia	DSM 14969 (=CIP 107471)	AF509824	*Acinetobacter towneri*
17A04^T	UN	Bendigo, Australia	DSM 14968^T (=CIP 107470^T)	AF509828	*Acinetobacter grimontii*
5B02	BG9	Bendigo, Australia	DSM 14965 (=CIP 107467)	AF509831	
7B02	UN	Bendigo, Australia	DSM 14966 (=CIP 107466)	AF509826	*Acinetobacter tjernbergiae*
7N16^T	UN	Bendigo, Australia	DSM 14971^T (=CIP 107465^T)	AF509825	*Acinetobacter tjernbergiae*
9A01^T	UN	Bendigo, Australia	DSM 14967^T (=CIP 107464^T)	AF509829	*Acinetobacter gerneri*

PCR amplification and sequencing of 16S rDNA. PCR amplifications of the 16S rDNA were carried out using the universal primers 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1525R (5′-AGAAAGGAGGTGATCCAGCC-3′), and the PCR protocol of Patel *et al.* (1995). PCR products were purified using the Concert Rapid PCR Purification system (Life Technologies) and subsequently cloned into the pGEM-T Easy Vector system II (Promega) in accordance with the manufacturers' instructions. Plasmids extracted from the resulting clones using QIAprep Spin Miniprep kits (Qiagen) were digested with *Eco*RI and run on a 1 % agarose gel to ensure that the plasmid contained the appropriate insert (approx. 1500 bases) before sequencing. All sequencing was carried out with an ABI DNA sequencer model 377a (Applied Biosystems) using Big-Dye Terminator kits (Applied Biosystems).

Phylogenetic analysis of strains. All phylogenetic analysis was carried out using programs available on BioManager by ANGIS (http://www.angis.org.au). The 16S rDNA sequences of the *Acinetobacter* strains were aligned with 16S rDNA sequences of all the known genomic species of *Acinetobacter* retrieved from GenBank using CLUSTAL W (Thompson *et al.*, 1994). Complete 16S rDNA sequences of *A. calcoaceticus* have been published by two different groups (Rainey *et al.*, 1994; Ibrahim *et al.*, 1997) and both of these sequences were included in the analysis. The 16S rDNA sequences of *Psychrobacter immobilis* and *Moraxella lacunata* were included as outgroups. The resulting multiple sequence alignment was corrected manually using the program DNASTAR, and approximately 200 bases at the 5′ end of the sequence were omitted from further analysis due to alignment ambiguities. Pairwise evolutionary distances were then computed from a continuous stretch of 1325 bases and a distance matrix was calculated with DNADIST (using the Jukes–Cantor correction parameter). Phylogenetic analysis was carried out by applying the neighbour-joining, parsimony and maximum-likelihood algorithms to ensure coherency of the clusters formed. Bootstrapping was performed (1000 replications) using the SEQBOOT program (Felsenstein, 1989) for the neighbour-joining and parsimony methods to check stability of the clusters formed.

DNA–DNA hybridization. The method used was the non-radioactive colorimetric method described by Ziemke *et al.* (1998). Comparative DNA–DNA hybridizations were carried out between this method and that described by Grimont *et al.* (1980) with previously characterized genomic species of *Acinetobacter*, i.e. *A. calcoaceticus* ATCC 23055^T, genomic species 3 (ATCC 19004), *A. junii* ATCC 17908^T, *A. johnsonii* ATCC 17909^T, genomic species 10 (ATCC 17924) and BG11 (ATCC 11171).

Phenotypic characterization. The type strains of the known genomic species were included in these phenotypic characterizations. All tests were carried out at 30 °C unless otherwise indicated. Growth at 37, 41 and 44 °C, haemolysis of horse blood and production of acid from glucose were performed as described previously (Bouvet & Grimont, 1986). Gelatin hydrolysis was carried out using the Microbact 24E identification system (Oxoid). Growth on DL-lactate, DL-4-aminobutyrate, *trans*-aconitate, citrate, glutarate, aspartate, *β*-alanine, L-histidine, D-malate, malonate, histamine, L-phenylalanine, phenylacetate, L-arginine (Bouvet & Grimont, 1986), L-tryptophan and 4-hydroxybenzoate (Bouvet & Jeanjean, 1989) was tested using the inorganic medium 'M70' of Veron (1975). All substrates were added at a final concentration of 0·1 % (w/v) and isolates were scored for growth after 2 and 6 days. All strains were also characterized phenotypically using the tests and methods detailed by Kämpfer *et al.* (1993). In some cases, these tests were the same as those described above and provided a check on the reproducibility of these characterizations. Carbon source assimilation patterns for the activated sludge strains and the known genomic species of *Acinetobacter* were determined using the Biolog GN Identification system (Oxoid) and these were obtained according to the manufacturer's instructions. Numerical taxonomic analysis of the Biolog data was performed using NTSYS-PC version 1.80 (Exeter software). Tests in which all strains examined were all positive or negative were excluded from this exercise.

Selection of the most discriminatory phenotypic characteristics for identification of strains of *Acinetobacter* from activated sludge. The activated sludge strains sharing greater than 70·0 % DNA similarity with one another were grouped together and treated as genomic species for selection of the most discriminatory phenotypic tests. The most discriminatory characteristics were selected from the complete matrix of Kämpfer *et al.* (1993) with the additional data for *A. ursingii*, *A. schindleri* and the other activated sludge isolates from this study. These tests were selected from the characteristics used with the CHARSEP program of Sneath (1979a), which determines the value of each characteristic as a potential separator of groups in an identification matrix. From the different separation indices obtained with CHARSEP, the vsP index (variance of separation potential) was then chosen to find those characteristics best able to differentiate between groups. A high vsP index for a particular characteristic indicates its usefulness. For the identification matrix, 32 tests were selected by CHARSEP and further evaluated using DIACHAR software (Sneath, 1980). A theoretical evaluation of the identification matrix was undertaken using MATIDEN (Sneath, 1979b), which calculates an identification score for a set of characteristics based on a Willcox probability score (Willcox *et al.*, 1973).

FISH analysis of *Acinetobacter* isolates. The two oligonucleotide FISH probes described by Wagner *et al.* (1994) and Snaidr *et al.* (1997) for the genus *Acinetobacter* were tested against these *Acinetobacter* strains using the same conditions of stringency for each probe as described in the original publications. Pure cultures were fixed in 4 % paraformaldehyde (Amann, 1995) and all subsequent FISH procedures incorporating the appropriate controls were performed according to Amann (1995). Probe EUB 338 of Amann *et al.* (1990) was used as a positive control to eliminate the possibility of false negative results from problems of probe permeabilities.

RESULTS AND DISCUSSION

Phylogenetic relationships between *Acinetobacter* strains using 16S rDNA sequence analyses

Almost-complete 16S rDNA sequences were acquired for the selected 13 *Acinetobacter* strains. The overall pattern of clustering seen generally agreed with the clusters obtained for the known genomic species of *Acinetobacter* by Ibrahim *et al.* (1997). The groupings of the activated sludge isolates here largely support those revealed from the phenotypic characterization data, in that they usually clustered separately from the described genomic species. For example, strains 17A04^T and 5B02 were 98·9 % similar to each other and less than 97·0 % similar to all other strains, with the exception of TU13, BG2, BG5 and BJ14, which showed similarity values greater than 97·0 %. Whereas strains 2N01 and AB1110^T were 97·1 % similar to each other, both were less than 97·0 % similar to all the other strains. Similarly, strains A7 and C5 were most similar to one another (98·1 %) and, with the exception of strain B2^T, they were less than 97·0 % similar to all other strains. Strain A23 was a distinct entity being less than 97·0 % similar to all of the other strains investigated. Strain 4N13^T was less than 97·0 % similar to all

of the other strains investigated except BJ15, with which it was 97·0 % similar. Strain 9A01^T showed a similarity of less than 97·0 % with all other strains except for the known genomic species BG10, BG11, TU13 and BG5, with which it shared similarity values of 97·0, 97·0, 97·1 and 97·0 % respectively. Strain 7N16^T was distinct from all other strains except 7B02, with which it was 98·2 % similar, whereas 7B02 shared a similarity value of greater than 97·0 % with a number of the known genomic species.

The phylogenetic tree (Fig. 1) generated using the maximum-likelihood algorithm reveals the relationships between all these strains. The following clusters were also observed in trees constructed using neighbour-joining and parsimony algorithms (data not shown). Strains A7, C5 and B2^T were always linked together, as were strains 5B02 and 17A04^T, AB1110^T and 2N01, and 7N16^T and 7B02. These clusters were each supported by high bootstrap values. The remainder of the activated sludge strains failed to cluster consistently with any other strains in trees constructed with the three algorithms. For example, with parsimony, strain 4B02^T clustered with A23 and 4N13^T, whereas when the neighbour-joining algorithm was used, 4B02^T emerged as a separate entity, and with maximum-likelihood, it clustered most closely with *A. schindleri*. Strain 9A01^T emerged separately with both maximum-likelihood and neighbour-joining, but linked with 17A04^T and 5B02 after parsimony. Also, strain A23 clustered most closely with 4N13^T with the parsimony and neighbour-joining algorithms. However, this clustering was not supported by high bootstrap values and Fig. 1 shows that both group separately, although A23 was most closely linked to BG10 and BG11.

DNA–DNA hybridization analysis

By themselves, the 16S rDNA sequence comparisons are not sufficiently discriminatory to enable speciation of these *Acinetobacter* isolates from activated sludge to be determined confidently (Stackebrandt *et al.*, 2002), since the similarity values were in most cases close to 97·0 %. Therefore DNA–DNA hybridizations were performed between appropriate strains with high 16S rDNA sequence similarities. The data (Table 2) clearly indicate that novel genomic species exist within these strains. For example, strains A7, B2^T and C5 had almost 100·0 % mutual DNA–DNA similarity, but less than 70·0 % similarity with any other strain, and therefore represent one novel species. Similarly, strains 7N16^T and 7B02 showed a mutual similarity value of 94·3 %. However, strains 5B02 and A23 shared DNA–DNA similarity values of greater than 70·0 % with some known genomic species, with 5B02 emerging as 88·1 % similar to *A. johnsonii*. Strain A23 was 89·2 % similar to genomic species 11 and, interestingly, 73·9 % similar to genomic species 10. The other isolates all had DNA similarities of less than 70·0 % to all other strains, including the known genomic species. Hence, strains 4N13^T, 17A04^T, 9A01^T and 4B02^T are each considered here to represent novel genomic species of *Acinetobacter*. Hybridizations

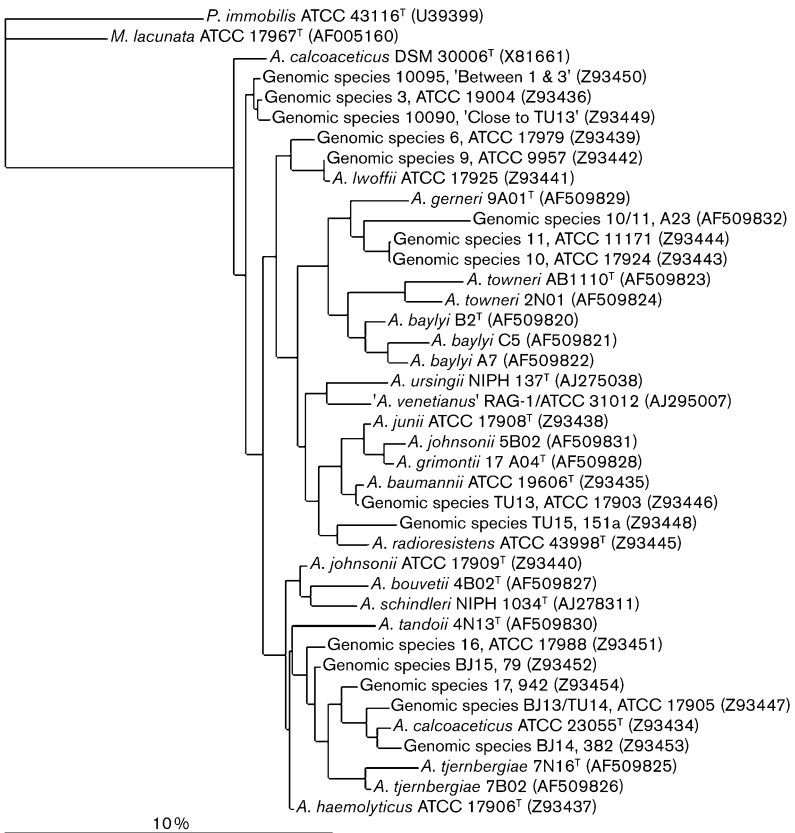

Fig. 1. Phylogenetic tree generated using the maximum-likelihood method of analysis of 16S rDNA sequences of *Acinetobacter* spp. including environmental strains from this study. BJ, genomic species described by Bouvet & Jeanjean (1989); TU, genomic species described by Tjernberg & Ursing (1989). The 16S rDNA sequences of *Psychrobacter immobilis* and *Moraxella lacunata* were included as outgroups. Bar, 10 % sequence divergence.

Table 2. DNA–DNA hybridization data for activated sludge isolates and currently described genomic species of *Acinetobacter*

All percentage values given here are mean values of at least two hybridization experiments. TU, genomic species described by Tjernberg & Ursing (1989). Strains: 1, *A. calcoaceticus* ATCC 17902; 2, *A. baumannii* strain 17035; 3, *Acinetobacter* genomic species 3, ATCC 17922; 4, *A. haemolyticus* ATCC 17906^T; 5, *A. junii* ATCC 17908^T; 6, *Acinetobacter* genomic species 6, ATCC 17979; 7, *A. johnsonii* strain 68; 8, *A. lwoffii* ATCC 15309^T; 9, *Acinetobacter* genomic species 10, ATCC 17924; 10, *Acinetobacter* genomic species 11, ATCC 11171; 11, *A. radioresistens* IAM 13186^T; 12, *Acinetobacter* genomic species 13, ATCC 17905; 13, *Acinetobacter* genomic species 14, strain 382; 14, *Acinetobacter* genomic species 15, strain 79; 15, *Acinetobacter* genomic species 16, ATCC 17988; 16, *Acinetobacter* genomic species 17, strain 942; 17, *Acinetobacter* genomic species 13 (TU), ATCC 17903; 18, *Acinetobacter* genomic species 14 (TU), strain 71; 19, *Acinetobacter* genomic species 15 (TU), strain 151a; 20, *A. schindleri* NIPH 1034^T; 21, *A. ursingii* NIPH 137^T.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
A. towneri AB1110^T/2N01	26	24	36	37	29	43	48	33	21	17	19	35	37	30	53	37	18	29	40	19	18
A. baylyi B2^T/C5/A7	49	32	42	30	27	36	57	25	21	11	13	30	27	29	32	35	20	24	29	13	23
A. gerneri 9A01^T	14	14	30	22	28	28	45	25	22	11	16	28	24	30	30	40	20	18	26	14	23
A. tjernbergiae 7N16^T/7B02	40	12	7	53	20	16	46	41	5	46	20	45	47	39	43	53	19	27	38	9	15
A. tandoii 4N13^T	28	25	36	20	30	36	26	29	46	13	17	33	25	28	39	40	23	22	33	15	22
A. grimontii 17A04^T	26	18	33	31	63	44	22	20	36	9	15	40	34	33	49	45	21	25	29	10	19
Acinetobacter strain A23	32	54	43	30	30	61	19	34	74	89	13	35	39	44	61	67	32	34	55	12	15
Acinetobacter strain 5B02	38	30	56	30	51	52	88	43	41	38	32	43	14	26	32	32	18	22	34	42	28
A. bouvetii 4B02^T	30	8	4	36	7	1	33	6	3	34	26	9	5	3	4	8	26	37	24	14	21
Pooled SD (%)	8·4	6·0	2·8	2·7	16·9	7·3	7·1	5·1	7·8	2·4	1·4	3·9	4·4	6·4	4·6	2·4	3·6	3·5	4·4	6·6	9·6

between genomic species 1 and 3, genomic species 5 and 7, and genomic species 10 and 11 revealed that the protocol of Ziemke *et al.* (1998) always gave higher values, in some cases by more than 20 %, than those obtained with the method of Grimont *et al.* (1980). For example, in an earlier study by Bouvet & Grimont (1986), the DNA similarities between genomic species 5 and 7 and genomic species 10 and 11 were shown to be 6–26 % and 22–30 %, respectively, whereas in this study the DNA similarities between these groups were shown to be 41·5 % (genomic species 5 and 7) and 55·6 % (genomic species 10 and 11). The conservative nature of the DNA–DNA hybridization method used in this study has been observed in other studies (Kämpfer *et al.*, 2002). Because this method of DNA–DNA hybridization is more conservative than other methods used in other studies with *Acinetobacter* (e.g. Bouvet & Grimont, 1986), there is added confidence in these taxonomic interpretations.

General characteristics of isolates

All strains were Gram-negative, oxidase-negative, strictly aerobic bacteria. On R2A agar, all grew as coccobacilli in pairs, as expected for members of the genus *Acinetobacter*. However, in some liquid media, including nutrient broth (Oxoid), morphology was pleiomorphic. For example, in some strains, including 2N01, individual cells appeared as elongated filaments that were sometimes in chains. Strains like AB1110^T, which were originally isolated by micromanipulation from activated sludge as large cell clusters, did not adopt this arrangement in pure culture in liquid or solid media, whereas several others that were not originally isolated as clusters did adopt this arrangement. In others, the individual coccobacilli were in chains, appearing like Eikelboom type 1863, a morphological form which some *Acinetobacter* spp. are known to assume in activated sludge plants (Seviour *et al.*, 1997).

Phenotypic characteristics of isolates

With the phenotypic characterization methods of Kämpfer *et al.* (1993), all strains tested gave positive results for assimilation of fumarate, L-malate and pyruvate, and all hydrolysed L-alanine-*p*-nitroanilide (L-alanine-pNA). None of the activated sludge isolates haemolysed horse blood, hydrolysed gelatin or grew at 44 °C. None of the strains tested produced acid from D-sucrose, D-mannitol, dulcitol, salicin, D-maltose, D-trehalose, methyl β-D-xyloside and *m*-erythritol. None grew on D-galactose, D-glucosaminic acid, D-sucrose, D-trehalose, D-turanose, *m*-erythritol, i-inositol, maltitol, L-glycine or L-lysine or hydrolysed *p*-nitrophenyl-β-D-galactopyranoside (pNP-β-D-galactopyranoside), pNP-β-D-glucuronide, pNP-α-D-glucopyranoside or pNP-β-glucopyranoside. Variable results were observed for the remainder of the tests.

All of the activated sludge isolates except strains 7B02 and 7N16^T grew at 37 °C. Strains C5, A7 and B2^T were very similar phenotypically except that strain A7 utilized L-histidine and hydrolysed L-proline-pNA, whereas the other two strains did

not. Strains 2N01 and AB1110^T also had many phenotypic characteristics in common, with the only differences seen in their abilities to grow on isovalerate, fumarate, DL-malate, L-malate and malonate. Similarly, strains 7B02 and 7N16^T shared most phenotypic characteristics.

Identifying these *Acinetobacter* strains

Because of their known high level of phenotypic similarity, some genomic species [the *Acb* complex of 1, 2, 3 and 13 (TU), and BG8 and BG9] were grouped together for construction of the identification matrix. On the basis of their vsP index, 32 tests with vsP scores of 64–95 % [far exceeding the >25 % recommended by Sneath (1979a)] were selected (Table 3). These tests were evaluated with DIACHAR and all 217 strains tested against the identification matrix with MATIDEN. Of these 217 strains, 187 (86·2 %) were correctly identified with a Willcox probability of more than 99·0 %, including all the activated sludge strains characterized here.

Biolog characterization of isolates

All 13 isolates used Tweens 40 and 80, methyl pyruvate and succinic acid. None of the 13 strains examined could utilize *m*-inositol, α-D-lactose, lactulose, L-fucose, D-psicose, D-raffinose, D-sorbitol, D-trehalose, turanose, xylitol, D-galacturonic acid lactone, D-glucosaminic acid, D-glucuronic acid, L-ornithine, D-serine, L-serine, thymidine, L-alanyl glycine, glycyl-L-aspartic acid, glycyl-L-glutaric acid, inosine, uridine, thymidine, DL-α-glycerol phosphate, glucose 1-phosphate or glucose 6-phosphate. All these phenotypic data were subjected to numerical analysis and are represented as a dendrogram (Fig. 2); the activated sludge isolates, with the single exception of strain A23, which groups closely with BG11, appeared to cluster separately from the described genomic species. This is most clearly shown in the case of strains A7, B2^T and C5. Most of the strains included in this study could not be identified with the Biolog system, repeating the experiences of Knight *et al.* (1993) with their activated sludge isolates. Good correlation was found between the Biolog GN identification system and the microplate method of Kämpfer *et al.* (1993) when utilization of the same substrate by the activated sludge strains was tested with both methods. However, small discrepancies were noted. For example, none of the activated sludge strains were shown to assimilate D-mannose using the method of Kämpfer *et al.* (1993), whereas Biolog showed that strains belonging to *A. baylyi* gave a positive result for this substrate. Similarly, all strains failed to utilize putrescine by the method of Kämpfer *et al.* (1993), whereas 4N13^T gave a positive result with Biolog.

FISH analysis of strains

The isolation of these previously undescribed genomic species of *Acinetobacter* from activated sludge systems raised the possibility that the FISH probes currently available for their *in situ* identification may not embrace these. It was considered possible that quantitative studies on their prevalence in activated sludge using these probes (Wagner *et al.*, 1994; Snaidr *et al.*, 1997) have been underestimating the total numbers of members of this genus. However, when these novel strains were probed with the ACA 23A probe of Wagner *et al.* (1994), all fluoresced, although not all strains fluoresced with the ACA 652b probe of Snaidr *et al.* (1997). Hence, theoretically these novel strains would not have been missed in the earlier study of Wagner *et al.* (1994), which suggested that members of this genus were not participating significantly in the process of microbiological phosphorus removal.

Therefore, this polyphasic approach with strains selected on the basis of their distinctive 16S–23S rDNA intergenic fingerprinting patterns (Carr *et al.*, 2001b) and a combination of phenotypic and genotypic characterization methods, including DNA–DNA hybridization, lends support to the view that strains AB1110^T and 2N01, B2^T, C5 and A7, and 7N16^T and 7B02 are novel genomic species of *Acinetobacter*. Likewise, all the data presented here provide evidence that 9A01^T and 4N13^T are taxonomically distinct from all other strains investigated. Strain 4B02^T was also shown to represent a novel genomic species according to DNA–DNA hybridization data. From the 13 activated sludge isolates examined, only two were found to belong to already described genomic species of *Acinetobacter*. Thus, strain A23 gave a DNA similarity value of greater than 70·0 % with both BG10 and BG11, although it showed a higher DNA homology with BG11, agreeing with both the 16S rDNA sequence data and the phenotypic data. According to the 16S–23S rDNA spacer region fingerprinting (Carr *et al.*, 2001b), this strain was most similar to BG11. Although strain 5B02 was 98·9 % similar to strain 17A04^T after 16S rDNA sequence analysis, DNA–DNA hybridization revealed it was greater than 70·0 % similar to *A. johnsonii*. Phenotypic data did not support a close relationship with either strain 17A04^T or *A. johnsonii*. It is interesting that *A. johnsonii* is reportedly unusual among the recognized genomic species of *Acinetobacter* in being unable to grow at 37 °C (Bouvet & Grimont, 1986) yet, in this study, 5B02 was able to do this. Despite this, the DNA–DNA hybridization data mean that 5B02 can be classified as *A. johnsonii* according to the species definition of Stackebrandt *et al.* (2002). Strain 17A04^T was also considered to represent a novel species. Hence, on the basis of the data presented here, seven novel species of *Acinetobacter* are proposed and are described below. In some cases, only single isolates are available for these species. Christensen *et al.* (2001) have recommended that novel species descriptions should be based on at least five isolates, a recommendation encouraged by Stackebrandt *et al.* (2002). However, it is felt that this should not be enforced and may not be appropriate for all bacteria. It could markedly reduce effective communication between bacterial systematists since the acquisition of these strains may take considerable time and may never be achieved.

It is clear from other studies that interspecies relationships in the genus *Acinetobacter* are not clearly defined, as many of

Table 3. Characteristics ($n=32$) that differentiate between all the currently described *Acinetobacter* genomic species and the activated sludge isolates

Based on the whole matrix published by Kämpfer *et al.* (1993) supplemented with the data for *A. ursingii* and *A. schindleri* and the genomic species described in this study selected by CHARSEP (Sneath, 1979a) and DIACHAR (Sneath, 1980). Apart from numbers of strains, figures in the table are the percentages of strains giving a positive result. BG, genomic species described by Bouvet & Grimont (1986); BJ, genomic species described by Bouvet & Jeanjean (1989); TU, genomic species described by Tjernberg & Ursing (1989). Strains: 1, *Acb* complex; 2, BG4; 3, BG5; 4, BG6; 5, BG7; 6, BG8/9; 7, BG10; 8, BG11; 9, BG12; 10, BJ14; 11, BJ15; 12, BJ16; 13, BJ17; 14, TU14; 15, TU15; 16, *A. ursingii*; 17, *A. schindleri*; 18, *Acinetobacter* strain 10090; 19, *Acinetobacter* strain 10095; 20, A7/C5/B2^T; 21, A23; 22, 9A01^T; 23, 4B02^T; 24, 5B02; 25, 7B02/7N16^T; 26, AB1110/2N01^T; 27, 17A04^T; 28, 4N13^T.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Number of strains	73	16	21	2	18	23	3	7	22	2	1	1	1	4	2	3	3	1	1	3	1	1	1	1	2	2	1	1
Acid production from:																												
D-Glucose	89	100	5	100	0	43	100	0	77	50	0	100	100	100	100	0	0	100	100	100	0	100	0	0	0	0	0	0
Lactose	88	88	0	100	0	9	67	0	5	50	0	100	100	100	50	0	0	100	100	100	0	100	0	0	0	0	0	0
L-Arabinose	89	100	0	100	11	91	100	0	95	50	0	100	100	100	100	0	0	100	100	100	0	100	0	0	0	0	0	0
L-Rhamnose	89	62	0	50	0	4	67	0	0	50	0	100	0	0	50	0	0	100	100	100	0	100	0	0	0	0	0	0
D-Xylose	89	100	5	100	6	74	100	0	77	50	0	100	100	100	100	0	0	100	100	100	0	100	0	0	0	0	0	0
D-Cellobiose	89	94	0	100	0	9	67	0	0	50	0	100	100	100	50	0	0	100	100	100	0	100	0	0	0	0	0	0
α-D-Melibiose	89	100	0	100	6	74	100	0	82	50	0	100	100	100	100	0	0	0	0	0	0	0	0	0	0	0	0	0
D-Mannose	88	100	0	100	0	48	100	0	55	50	0	100	100	100	100	0	0	100	100	100	0	100	0	0	0	0	0	0
Assimilation of:																												
Pimelate	99	56	57	100	61	91	100	100	100	50	0	100	100	75	100	100	67	100	100	100	100	100	0	0	0	0	0	0
cis-Aconitate	95	88	14	100	6	0	33	0	0	50	0	100	0	0	0	33	100	100	100	100	100	0	0	0	0	0	0	100
trans-Aconitate	93	69	0	0	0	0	33	0	0	50	0	0	100	0	0	0	33	100	100	100	100	0	0	0	0	0	0	100
Adipate	97	44	71	100	56	87	67	100	100	50	0	100	100	100	100	100	0	0	100	100	100	100	0	0	0	0	0	0
4-Aminobutyrate	100	94	81	50	56	78	67	86	100	50	0	100	0	0	50	0	0	0	0	100	100	100	0	0	0	0	100	100
Azelate	97	0	0	0	17	83	67	100	95	50	0	100	0	0	50	100	67	100	100	100	100	100	0	0	0	0	0	0
Citrate	100	69	48	100	56	9	100	57	0	100	0	100	100	100	0	100	33	100	100	100	100	100	0	100	0	0	100	0
Glutarate	97	12	14	50	28	26	100	100	100	50	0	100	100	50	50	100	0	100	100	0	100	100	100	0	0	0	0	0
Malonate	92	75	57	100	50	65	0	14	100	100	100	0	100	75	50	0	33	0	100	100	0	0	0	0	0	50	0	100
Oxoisocaprate	100	100	33	100	50	22	0	0	100	100	100	100	100	25	0	0	0	100	100	0	0	0	0	0	0	0	0	0
Suberate	100	12	71	50	50	100	100	100	100	50	0	100	0	50	100	100	67	0	100	100	100	100	0	0	0	0	0	0
β-Alanine	93	6	0	0	0	0	100	100	0	100	100	0	100	50	0	0	0	0	100	0	100	100	0	0	0	0	0	0
L-Arginine	100	100	95	100	33	4	0	0	95	100	100	100	100	75	0	0	0	0	0	100	0	0	0	0	100	0	0	100
L-Aspartate	97	38	29	100	61	0	100	100	27	0	0	100	0	50	0	0	0	100	100	100	100	0	0	0	0	0	0	100
DL-Aspartate	99	6	10	100	44	0	100	86	0	50	0	0	0	0	0	67	100	100	100	100	100	0	100	0	50	0	0	100
L-Glutamate	100	100	100	100	100	39	100	100	100	100	100	100	100	100	50	0	0	0	0	0	0	0	0	0	0	0	0	0
L-Histidine	100	100	95	100	0	0	100	100	0	100	100	100	100	100	0	0	0	100	100	33	100	0	100	0	100	0	100	100
L-Leucine	99	94	29	100	17	0	0	0	100	100	100	100	100	25	0	0	0	0	100	0	0	0	0	0	0	0	0	0
L-Phenylalanine	82	0	0	0	0	0	0	0	100	100	100	100	100	100	50	0	0	100	100	0	0	100	0	0	0	0	0	100
L-Tryptophan	93	0	0	0	0	0	0	0	14	100	100	0	100	100	0	0	0	100	100	0	0	0	0	0	0	0	0	0
L-Leucinamide	100	88	10	100	22	0	0	0	100	100	100	0	100	25	0	0	0	0	0	0	0	0	0	0	50	0	0	0
4-Hydroxybenzoate	95	100	0	50	6	0	67	86	9	100	100	0	100	100	0	100	67	100	100	100	0	100	0	0	0	0	0	0
Phenylacetate	85	0	0	0	0	83	0	71	95	100	100	100	100	100	100	0	0	100	100	100	0	100	0	0	0	0	0	0
Quinate	95	100	0	100	56	0	67	100	0	50	100	100	100	100	0	100	0	0	100	100	100	0	100	0	100	0	0	0

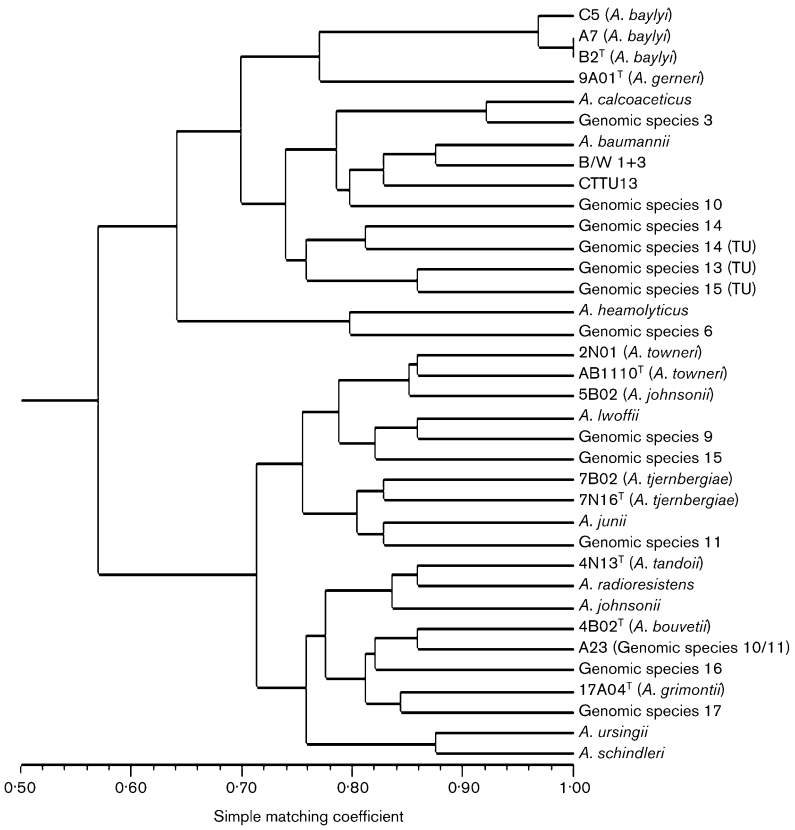

Fig. 2. Dendrogram generated from Biolog data using the UPGMA algorithm as determined by the simple matching coefficient and UPGMA clustering. TU, genomic species described by Tjernberg & Ursing (1989).

the genomic species are phenotypically very similar to one another and cannot be readily differentiated (Gerner-Smidt *et al.*, 1991). This 'blurring' of speciation complicates species delineation. However, sequencing of appropriate housekeeping genes (Stackebrandt *et al.*, 2002), which ideally requires complete genome sequencing, may assist in this task, but until then attempts to understand the ecology and taxonomy of this organism will continue to frustrate.

It is worthy of mention that these novel strains were all isolated from a very small number of activated sludge plants over a relatively short time period and it is difficult not to conclude that a more extensive search of a larger number of geographically widely distributed plants would reveal many more undescribed members of this genus.

Description of *Acinetobacter baylyi* sp. nov.

Acinetobacter baylyi (bay.ly′i. N.L. masc. gen. n. *baylyi* in honour of Ronald Bayly, an Australian microbiologist who has contributed to the understanding of the physiology of this genus).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). Growth occurs at 37 and 41 °C, but not at 44 °C. Acid is produced from D-glucose, horse blood is not haemolysed and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), pimelate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, malonate, suberate, L-arginine, L-aspartate, DL-aspartate, 4-hydroxybenzoate, phenylacetate and quinate are all utilized, whereas L-histidine is utilized by some strains. Oxoisocaprate, glutarate, β-alanine, L-glutamate, L-leucine, L-phenylalanine, L-tryptophan and L-leucinamide are not utilized and acid is not produced from α-D-melibiose.

The type strain is B2^T (=DSM 14961^T=CIP 107474^T); it was isolated from activated sludge. This strain does not utilize L-histidine.

Description of *Acinetobacter tjernbergiae* sp. nov.

Acinetobacter tjernbergiae (tjern.ber.gi′ae. N.L. fem. gen. n. *tjernbergiae* in honour of Ingela Tjernberg, a Swedish microbiologist and taxonomist who has contributed to our understanding of the taxonomy of this genus).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). No growth occurs at 37 °C or higher. Acid is not produced from D-glucose, horse blood is not haemolysed and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), L-arginine, L-histidine and quinate are all used as sole sources of carbon and energy and some strains utilize DL-aspartate and L-leucinamide. *cis*-Aconitate, pimelate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, glutarate, malonate, oxoisocaprate, suberate, β-alanine, L-aspartate, L-glutamate, L-leucine, L-phenylalanine,

L-tryptophan, 4-hydroxybenzoate and phenylacetate are not utilized.

The type strain is 7N16^T (= DSM 14971^T = CIP 107465^T); it was isolated from activated sludge. This strain does not use DL-aspartate or L-leucinamide.

Description of *Acinetobacter towneri* sp. nov.

Acinetobacter towneri (tow.ner′i. N.L. masc. gen. n. *towneri* in honour of Kevin Towner, an English microbiologist who has contributed to our understanding of the genetics of this genus).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). Growth occurs at 37 and 41 °C, but not at 44 °C. No acid production from D-glucose, no haemolysis of horse blood and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), DL-lactate and pyruvate are utilized as the sole sources of carbon and energy. Most strains utilize malonate, L-malate, DL-malate, fumarate and isovalerate. Pimelate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, glutarate, oxoisocaprate, suberate, β-alanine, L-arginine, L-aspartate, DL-aspartate, L-glutamate, L-histidine, L-leucine, L-phenylalanine, L-tryptophan, L-leucinamide, 4-hydroxybenzoate, phenylacetate and quinate are not utilized.

The type strain is AB1110^T (= DSM 14962^T = CIP 107472^T); it was isolated from activated sludge. This strain utilizes isovalerate, fumarate, DL-malate, L-malate and malonate.

Description of *Acinetobacter bouvetii* sp. nov.

Acinetobacter bouvetii (bou.vet.i′i. N.L. masc. gen. n. *bouvetii* in honour of Philippe Bouvet, a French microbiologist who has contributed to our understanding of the taxonomy of this genus).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). Growth occurs at 37 and 41 °C, but not at 44 °C. Acid is not produced from glucose, horse blood is not haemolysed and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), glutarate, DL-aspartate, L-histidine and quinate are utilized as sole sources of carbon and energy. Pimelate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, malonate, oxoisocaprate, suberate, β-alanine, L-arginine, L-aspartate, L-glutamate, L-leucine, L-phenylalanine, L-tryptophan, L-leucinamide, 4-hydroxybenzoate and phenylacetate are not utilized.

The type strain is 4B02^T (= DSM 14964^T = CIP 107468^T); it was isolated from activated sludge.

Description of *Acinetobacter grimontii* sp. nov.

Acinetobacter grimontii (gri.mon.ti′i. N.L. masc. gen. n. *grimontii* in honour of Patrick Grimont, a French microbiologist who has contributed to our understanding of the taxonomy of this genus).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). Growth occurs at 37 and 41 °C, but not at 44 °C. Acid is not produced from glucose, horse blood is not haemolysed and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), 4-aminobutyrate, citrate and L-histidine are utilized as sole sources of carbon and energy. Pimelate, *trans*-aconitate, *cis*-aconitate, adipate, azelate, glutarate, malonate, oxoisocaprate, suberate, β-alanine, L-arginine, L-aspartate, DL-aspartate, L-glutamate, L-leucine, L-phenylalanine, L-tryptophan, L-leucinamide, 4-hydroxybenzoate, phenylacetate and quinate are not utilized.

The type strain is 17A04^T (= DSM 14968^T = CIP 107470^T); it was isolated from activated sludge.

Description of *Acinetobacter gerneri* sp. nov.

Acinetobacter gerneri (ger.ner′i. N.L. masc. gen. n. *gerneri* in honour of Peter Gerner-Smidt, a Danish microbiologist who has contributed to our knowledge of the taxonomy of this genus).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). Growth occurs at 37 and 41 °C, but not at 44 °C. Acid is produced from D-glucose, horse blood is not haemolysed and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), pimelate, adipate, 4-aminobutyrate, azelate, citrate, glutarate, suberate, β-alanine, L-phenylalanine, L-tryptophan, 4-hydroxybenzoate and phenylacetate are all utilized. *cis*-Aconitate, *trans*-aconitate, malonate, oxoisocaprate, L-arginine, L-aspartate, DL-aspartate, L-glutamate, L-histidine, L-leucine, L-leucinamide and quinate are not utilized.

The type strain is 9A01^T (= DSM 14967^T = CIP 107464^T); it was isolated from activated sludge.

Description of *Acinetobacter tandoii* sp. nov.

Acinetobacter tandoii (tan.do′i.i. N.L. masc. gen. n. *tandoii* in honour of Valter Tandoi, an Italian bacteriologist who has contributed to our understanding of *Acinetobacter* in activated sludge).

Characteristics correspond to those of the genus (Juni, 1984) and colonies on nutrient agar are as described for all other genomic species (i.e. circular, convex, smooth and slightly opaque). Growth occurs at 37 °C, but not at 41 or 44 °C. Acid is not produced from D-glucose, horse blood is not haemolysed and gelatin is not hydrolysed. Using the method of Kämpfer *et al.* (1993), *cis*-aconitate, *trans*-aconitate, 4-aminobutyrate, malonate, L-arginine, L-aspartate,

DL-aspartate, L-histidine and L-phenylalanine are all utilized. Pimelate, adipate, azelate, citrate, glutarate, oxoisocaprate, suberate, β-alanine, L-glutamate, L-leucine, L-tryptophan, L-leucinamide, 4-hydroxybenzoate, phenylacetate and quinate are not utilized.

The type strain is 4N13^T (=DSM 14670^T=CIP 107469^T); it was isolated from activated sludge.

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