Journal of Industrial Microbiology & Biotechnology (1997) 18, 267–271

Diversity of isolates of *Acinetobacter* from activated sludge systems based on their whole cell protein patterns

AM Maszenan, RJ Seviour, BM McDougall and JA Soddell

Biotechnology Research Centre, La Trobe University, Bendigo, Victoria 3550, Australia

Whole cell protein extracts from strains of the currently recognized genomic species of *Acinetobacter*, together with those from a range of isolates of several genomic species identified using the Biolog system and obtained from a biological nutrient-removal activated sludge plant were analysed by SDS-PAGE. The dendrograms obtained after numerical analysis for the known genomic species generally supported the taxonomic relationships suggested from earlier DNA–DNA hybridisation data. In some cases the activated sludge isolates identified to genomic species level clustered closely with the corresponding genomic species reference strains, although isolates 5 and 8/9 were scattered throughout the dendrogram. Considerable variations were seen in the protein patterns of the 27 different environmental isolates of genomic species 7 that were analysed. Three unidentified *Acinetobacter* isolates examined formed their own subcluster.

Keywords: *Acinetobacter*; activated sludge; phosphate removal; taxonomy

Introduction

The increasing importance of *Acinetobacter* isolates as serious nosocomial infectious organisms in hospitals around the world has encouraged attempts to resolve a confused taxonomy, especially interspecies delineation, and to develop reliable methods for isolate identification, as evidenced by the number of taxonomic papers presented at the Acinetobacter '94 conference in Edinburgh (1994). Characters selected for classifying and identifying these clinical isolates have included DNA–DNA hybridisation which forms the basis for their present groupings [4,5,28], plasmid profiles [8], ribotyping [10], cell envelope [6,7] and whole cell protein patterns [1], genomic fingerprinting [13], and several phenotypic identification schemes based on earlier suggestions of Bouvet and Grimont [4] and Bouvet and Jean-Jean [5], like those of Gerner-Smidt *et al* [11]. These clinical isolates appear to belong mainly to genomic species 2 and 3 of Bouvet and Grimont [4] and DNA group 13 of Tjernberg and Ursing [29], although other genomic species have also been isolated [9,11]. However, in none of these studies have all the isolates examined been completely satisfactorily classified or identified with any of the characters used, and the taxonomic relationships revealed for any group of isolates often differed according to the characters selected.

In addition to hospitals, *Acinetobacter* spp are found in other habitats, including biological nutrient-removal activated sludge plants [15,29], and far less is known about their interspecies relationships than the clinical isolates. However, genomic species 7 is often the main genomic species isolated, although others have also been detected [2,19]. Unfortunately the phenotypic schemes of Bouvet and Grimont [4] and Gerner-Smidt *et al* [9] and rapid kits like Biolog do not always lead to a reliable identification [19,27]. Consequently other methods have been sought to resolve the relationships among these environmental isolates and assess the extent of their genotypic and phenotypic diversity. PCR genomic fingerprinting using tRNA consensus primers and RAPD appear to provide a rapid and reproducible means for their identification, but again these identifications do not always agree with those obtained with other methods [31]. SDS-PAGE of whole cell proteins has been applied successfully to resolving intra-generic relationships with many Gram-positive and Gram-negative bacteria [24,30], and protein patterns seem to correlate well with DNA–DNA hybridisation data. Both Kampfer *et al* [17,18] and Bosch and Cloete [3] applied this technique to *Acinetobacter* isolates from activated sludge plants, but they examined only a few of the currently recognised genomic species and a small number of the environmental isolates, thus limiting the taxonomic value of their findings. Therefore, we assessed SDS-PAGE of proteins as a taxonomic character to see if it can discriminate between all the currently recognised genomic species of Bouvet and Grimont [4], Bouvet and Jean-Jean [5] and Tjernberg and Ursing [28]. Attempts were also made to determine whether the technique could then be used to indicate diversity among particular genomic species of the environmental isolates which had been identified earlier in this laboratory using the Biolog identification scheme, and to determine if more genomic species than those currently recognised might exist on the basis of their protein patterns.

Materials and methods

Strains used in study

A total of 65 *Acinetobacter* isolates obtained from a full-scale University of Cape Town biological nutrient-removal plant over a 9-month period at Bendigo were cultured and identified to genus level using the transformation assay of Juni [16] as described earlier [2,20]. They were then ident-

Correspondence: Dr RJ Seviour, Biotechnology Research Centre, La Trobe University, Bendigo, Victoria 3550, Australia
Received 6 September 1996; accepted 10 December 1996

ified to genomic species level, wherever possible, using the Biolog GN system [19] and maintained at −80°C. Also included in this study was a strain of each of the 17 genomic species of Bouvet and Grimont [4], kindly supplied by Professors RC Bayly, Monash University, Melbourne, Victoria, Australia and PJM Bouvet, Institut Pasteur, Paris, France and the DNA groups 13, 14 (the same as genomic species 13 of Bouvet and Grimont) and 15 of Tjernberg and Ursing [28] kindly provided by Prof I Tjernberg, Dept of Clinical Bacteriology, University of Lund, Malmo, Sweden. These strains are prefixed by B and T respectively, ie genomic species 1 [4] is expressed as B1 and so on.

Extraction and analysis of whole cell proteins

All strains were grown under standardised conditions [30]. Frozen cultures were resuscitated by incubation in nutrient broth (Gibco BRL, Melbourne, Australia) at 30°C for 48 h and then streaked onto tryptic soy agar (TSA) before being incubated again at 30°C for 48 h. A single colony was then subcultured onto a slope of TSA, incubated as before and then streaked onto nutrient agar (Gibco BRL), incubated at 30°C for 48 h and Gram-stained smears were checked for purity. Finally, a single colony was inoculated into 50 ml nutrient broth in a 250-ml Erlenmeyer flask and incubated at 30°C for 48 h at 180 rpm on an orbital incubator (Paton Industries, Adelaide, Australia). Cells were harvested by centrifugation (10 000 *g*) for 30 min, washed twice with milli Q water and resuspended in the 0.125 M Tris HCl buffer pH 6.8 containing 4% sodium dodecyl sulphate (SDS) and 20% (w/w) glycerol, used by Alexander *et al* [1] except that 20 mM dithiothreitol was used instead of mercaptoethanol. These cell suspensions (1 g cells ml^{-1} buffer) were heated in a water bath at 100°C for 15 min, centrifuged at 4°C for 3 h (16 000 *g*), and the supernatants containing the proteins were stored at −20°C and were then analysed by SDS-PAGE using the methods of Laemmli [21] and Jackman [14]. Gels consisting of 10% acrylamide and 2.7% bisacrylamide crosslinking were run at 100 V and 200 mA in a Pharmacia GE 2/4 LS apparatus with cooling, until the bromophenol blue marker dye reached to within 1 cm of the bottom of the gel. They were then fixed for 24 h in a methanolic trichloracetic acid solution, before being stained with Coomasie brilliant blue R250 and destained in a 25% ethanolic 8% acetic acid solution. All extracts were run at least in duplicate, and allowances were made for gel variability by running the same extracts on several gels. Because gel smearing was commonly seen with some extracts, and could not be eliminated completely by changes in experimental protocol, densitometric analyses of the patterns were not attempted, since they are not appropriate for gels with high and variable background colour [1]. Therefore, gel patterns were recorded by photography and accurate line diagrams were compared visually with each other and the original gels. Presence of the same extracts on different gels made cross-comparisons relatively straight-forward.

Patterns were then subjected to numerical analysis on the basis of individual band electrophoretic mobilities using NTSYS-pc version 1.8 (Exeter Software) with computation of simple matching coefficients S_{sm}. Cluster analysis was performed using the unweighted pair group method with the arithmetic averages (UPGMA) algorithm [26]. Protein bands with indistinguishable electrophoretic mobilities were assumed to be homologous and were scored accordingly.

Results

A total of 33 bands was scored on all the gels examined, although not all were present in each strain. Occasionally, marked differences in the intensity of individual bands were seen between strains, but these were not allowed for in the numerical analyses.

Protein profiles of the reference strains of the known genomic species

The dendrogram obtained after analyses of the patterns from B1–17 and T13–15 (Figure 1) reveals several relationships worthy of comment. Thus B1, B2, B3 and T13 all had a high similarity, clustering at an S_{sm} of 0.93, and in fact it was not possible to distinguish between B2 and T13 on the basis of their protein patterns. Furthermore, B13 and T14 as expected [28] were very similar to each other, as were B8 and B9, B15 and B16, and B10 and B11, while B12 showed the lowest similarity to all the other strains.

Protein profiles of activated sludge isolates identified as belonging to genomic species 7

The 27 genomic species 7 isolates selected for this study to represent those taken at different times during the study and from different operational zones of the activated sludge plant [20] gave a range of identification scores with Biolog. Although their SDS-PAGE protein patterns support the view that considerable phenotypic diversity can exist within individual genomic species (Figure 2), some strains gave identical patterns. In certain cases this was not wholly unexpected, as with strains 12BO2 and 12BO3, which were both obtained from the same sample of biomass on the same day. Such results also add confidence to the method used for analysis of these data. Furthermore, all of these activated sludge strains identified as genomic species 7

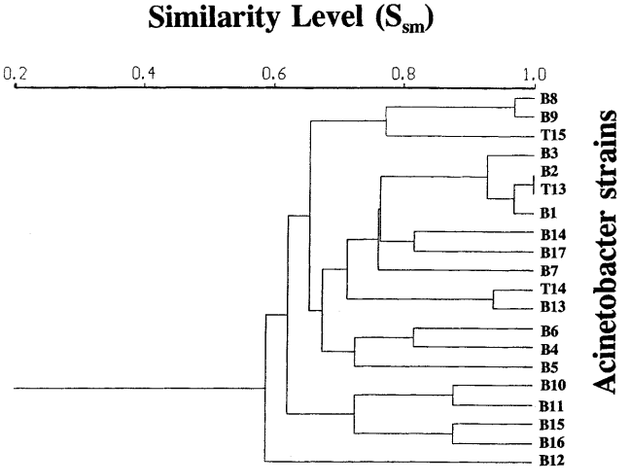

Figure 1 Dendrogram of reference strains of the known genospecies of *Acinetobacter* after S_{sm}-UPGMA numerical analyses of SDS-PAGE protein patterns.

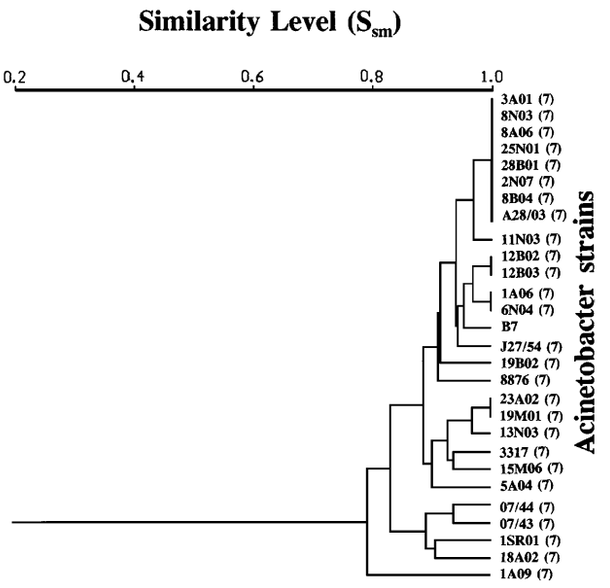

Figure 2 Dendrogram of isolates of genospecies of *Acinetobacter* obtained from a biological nutrient-removal plant, after S_{sm}-UPGMA numerical analyses of their SDS-PAGE protein profiles. B7 is the reference strain of genospecies 7.

clustered together as S_{sm} of 0.79, consistent with them belonging to the one genospecies [24,30].

Protein profiles of activated sludge isolates of Acinetobacter belonging to other genomic species

Although variations in protein patterns were also seen in the isolates of other genomic species examined, these often clustered closely with their corresponding type strain (Figure 3), as seen with most strains of genomic species 2, 3, 10, 11 and 12. However, there were some exceptions, and often these strains yielded low identification scores with Biolog (eg ISRO1). Most isolates identified as genomic species 5, 8 and 9 were more widely scattered throughout the dendrogram, and thus did not cluster closely with their respective type strains. The three strains 22A01, 7A05 and 12AO2, which could not be identified with Biolog, all grouped into a single cluster closest to B15 and 16 (Figure 3).

Discussion

Although several of the genomic species of *Acinetobacter* and a small number of isolates from activated sludge have been characterised previously by SDS-PAGE [3,17], the present study was designed to include all the currently recognised genomic species, and large numbers of environmental isolates of several known genomic species. One aim was to see if this technique, after calibration against known genomic species, could be used to identify reliably individual genomic species among such isolates. No perfect method exists for comparing gel patterns [24,30], but visual comparison was preferred here partly because of the background smearing on some gels.

Numerical analyses of the genomic type strain protein patterns revealed groupings similar to those obtained based on DNA/DNA homology. For example, the high mutual level of similarity seen here with B1, B2, B3 and T13 supports the view that these could all be amalgamated into a single group, the *Acinetobacter calcoaceticus-baumannii* complex [11,12,17,28]. Strains B8 and B9, indistinguishable phenotypically [4], also had very similar patterns, as did B10 and B11, B4 and B6, B15 and B16 and T14 and B13, while B12 was the most isolated of the strains. Most of these relationships agree with those based on the DNA homology estimates of Tjernberg and Ursing [28], except that their data suggested that B8 and T15 were more closely related to each other than to the rest.

However, other studies using different characters revealed different relationships between these genomic species. For example Rainey *et al* [25], on the basis of their 16SrDNA sequences suggested that B2 and B5 were most similar to each other, while B1, 4, 7 and 8 also clustered, although B12 still remained isolated. Also, RAPD PCR fingerprinting showed that B7 and B9 were very closely related [31], as were B15 and B16, and B14 and B17, while Nowak *et al* [23] also using PCR fingerprinting, but with different primers and region of the genome, obtained results which suggested genospecies 5, 7 and 10 were the same, while each of the others produced a unique band pattern.

Furthermore, the relationships between these known genomic species suggested from results obtained in this present study were different often from those proposed by Bosch and Cloete [3] with SDS-PAGE. Such interlaboratory comparisons are known to be difficult [24]. Bosch and Cloete [3] analysed only five genomic species, and their data clustered B1, B2 and B4 together, which might be a consequence of not including B3 and T13 in their study. However, their grouping supports the data of Nishimura *et al* [22], and their suggestion was that B2 and B4 should be considered subspecies of B1 (*A. calcoaceticus*). Bosch and Cloete [3] also found that B5 and B8 had very similar protein profiles, and some strains on analysis gave protein patterns sufficiently distinctive to warrant their recognition as new species. However, no DNA hybridisation data were presented to support such claims. As only single isolates of each of their type strains were analysed, the levels of diversity of patterns within each known genomic species were investigated.

Considerable diversity in the protein profiles of their activated sludge genomic species 7 isolates were noticed by Bosch and Cloete [3], results which agree with those obtained in this investigation, where some correlation between their Biolog identification score and clustering position was also apparent. The data presented here indicate that a unique pattern does not exist for each genomic species. However, in both studies, patterns of these isolates of genomic species 7 were sufficiently different, with one or two exceptions, from the rest of the isolates of other genomic species analysed to give a well-defined cluster and suggest that they may all belong to a single genomic species 7. Furthermore in most cases, these isolates clustered with the reference strain of B7.

In fact, many of the identified environmental isolates examined here clustered with their corresponding genomic type strains, although environmental isolates identified using Biolog as 5, 8 and 9 were more widely dispersed throughout the dendrogram. Whether some of these are

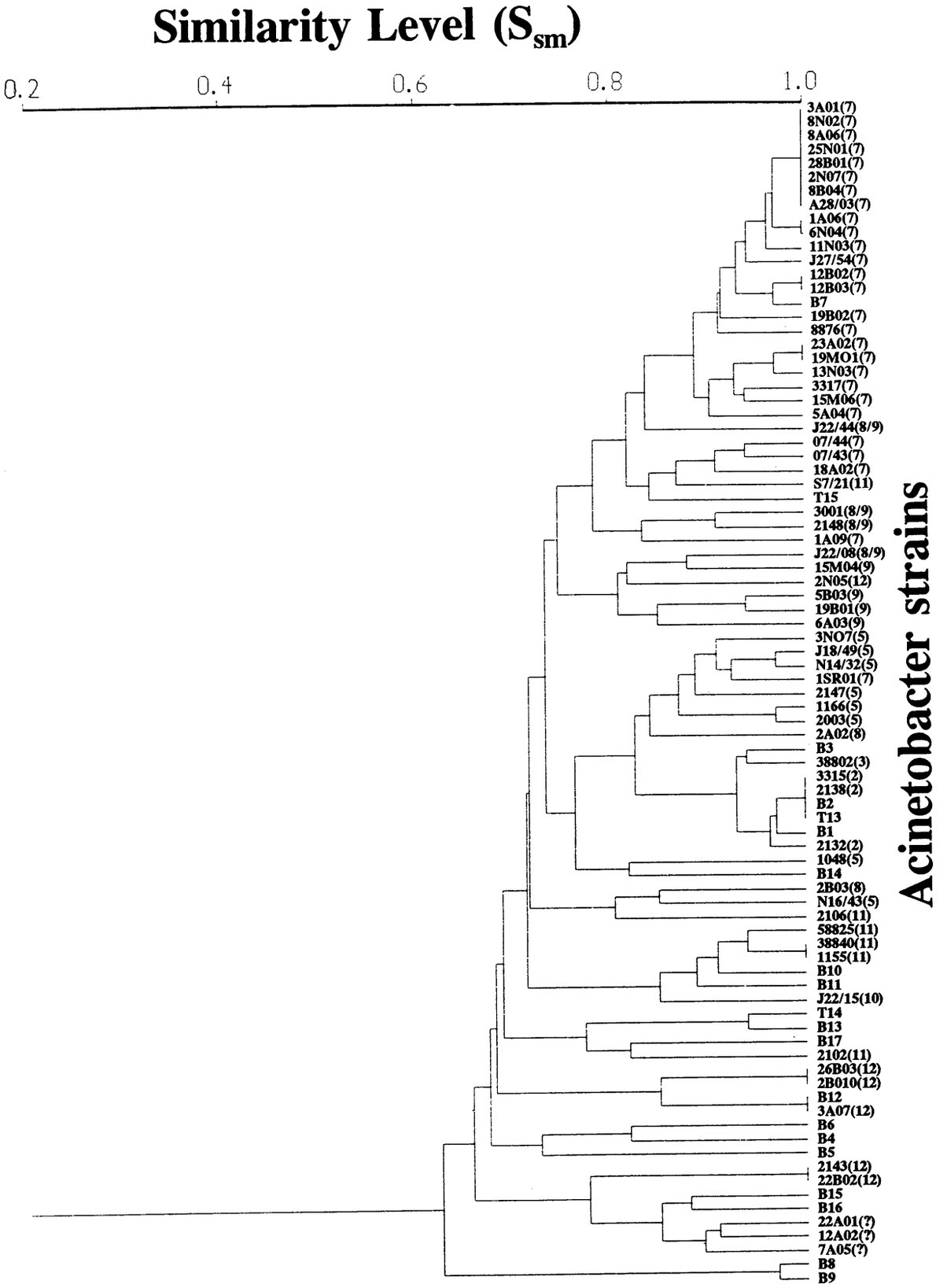

Figure 3 Dendrogram of all the *Acinetobacter* isolates including reference strains after numerical analyses of their SDS-PAGE protein profiles with the UPGMA algorithm. Numbers in parentheses refer to their genospecies identification. B and T prefixed strains are reference strains of Bouvet and Grimont [4] and Tjernberg and Ursing [28] respectively.

wrongly identified (eg 1048, 2102) needs to be verified using DNA homology studies, as do the taxonomic relationships of the three identified isolates 22A01, 12AO2 and 7AO5, which clustered together but sufficiently close to B15 and B16 to require further study. The limitations of Biolog as a reliable method for identifying *Acinetobacter* from activated sludge systems have been reported before [9].

Even in the absence of a unique or easily distinguished protein pattern for each genomic species in these activated sludge isolates, and the demonstrated heterogeneity of protein profiles within them, SDS-PAGE of whole cell proteins may still be a valuable aid in clarifying the taxonomy of such *Acinetobacter* isolates, but only when used in conjunction with other parameters including DNA homology. By itself it will not enable these environmental strains to be readily identified, and so studies on their ecology and natural distributions are still limited by inadequate methodology.

References

1 Alexander M, H Rahman, M Taylor and WC Noble. 1988. A study of the value of electrophoresis and other techniques for typing *Acinetobacter calcoaceticus*. J Hosp Infect 12: 273–287.
2 Beacham AM, RJ Seviour, KC Lindrea and I Livingston. 1990. Genospecies diversity of *Acinetobacter* isolates obtained from a biological nutrient removal plant of modified UCT plant configuration. Wat Res 24: 23–29.
3 Bosch M and TE Cloete. 1993. The identification and classification of *Acinetobacter* strains exhibiting variations in phosphate accumulation by SDS-PAGE and numerical taxonomy. Curr Microbiol 27: 169–175.
4 Bouvet PJM and PAD Grimont. 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.
5 Bouvet PJM and S Jean-Jean. 1989. Delineation of new proteolytic genomic species in the genus *Acinetobacter*. Res Microbiol 140: 291–299.
6 Bouvet PJM, S Jean-Jean, J-F Vieu and L Dijkshoorn. 1990. Species, biotype, and bacteriophage type determination compared with cell envelope protein profiles for typing *Acinetobacter* strains. J Clin Microbiol 28: 170–176.
7 Dijkshoorn L, HM Aucken, P Gerner-Smidt, ME Kaufmann, J Ursing and TL Pitt. 1993. Correlation of typing methods for *Acinetobacter* isolates from hospital outbreaks. J Clin Microbiol 31: 702–705.
8 Gerner-Smidt P. 1989. Frequency of plasmids in strains of *Acinetobacter calcoaceticus*. J Hosp Infect 14: 23–28.
9 Gerner-Smidt P, I Tjernberg and J Ursing. 1991. Reliability of phenotypic tests for identification of *Acinetobacter* species. J Clin Microbiol 29: 277–282.
10 Gerner-Smidt P. 1992. Ribotyping of *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. J Clin Microbiol 30: 2680–2685.
11 Gerner-Smidt P and W Frederiksen. 1993. *Acinetobacter* in Denmark: I. Taxonomy, antibiotic susceptibility, and pathogenicity of 112 clinical strains. Acta Path Microbiol Immunol Scand 101: 815–825.
12 Gerner-Smidt P and I Tjernberg. 1993. *Acinetobacter* in Denmark: II. Molecular studies of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. Acta Path Microbiol Immunol Scand 101: 826–832.
13 Graser Y, I Klare, E Halle, R Gantenberg, P Buchholz, HD Jacobi, W Presber and W Schonain. 1993. Epidemiological study of an *Acinetobacter baumannii* outbreak by using polymerase chain reaction fingerprinting. J Clin Microbiol 31: 2417–2420.
14 Jackman PJH. 1985. Bacterial taxonomy based on electrophoretic whole-cell protein patterns. In: Chemical Methods in Bacterial Systematics (Goodfellow M and DE Minnikin, eds), pp 115–128, Academic Press, London.
15 Jenkins D and V Tandoi. 1991. The applied microbiology of enhanced biological phosphate removal—accomplishments and needs. Wat Res 25: 1471–1478.
16 Juni E. 1972. Interspecies transformation of *Acinetobacter*: genetic evidence for a ubiquitous genus. J Bacteriol 101: 568–573.
17 Kampfer P, K Bark, H-J Busse, G Auling and W Dott. 1992. Numerical and chemotaxonomy of polyphosphate accumulating *Acinetobacter* strains with high polyphosphate : AMP phosphotransferase (PPAT) activity. Syst Appl Microbiol 15: 409–419.
18 Kampfer P, I Tjernberg and J Ursing. 1993. Numerical classification and identification of *Acinetobacter* genomic species. J Appl Bacteriol 75: 259–268.
19 Knight GC, SA McDonnell, RJ Seviour and JA Soddell. 1993. Identification of *Acinetobacter* isolates using the Biolog identification system. Lett Appl Microbiol 16: 261–264.
20 Knight GC, RJ Seviour, JA Soddell, SA McDonnell and RC Bayly. 1995. Metabolic variation among strains of *Acinetobacter* isolated from activated sludge. Wat Res 29: 2081–2084.
21 Laemmli UK. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227: 680–685.
22 Nishimura Y, H Kanzaki and H Iizuka. 1988. Taxonomic studies of *Acinetobacter* species based on the electrophoretic analysis of enzymes. J Basic Microbiol 28: 363–370.
23 Nowak A, A Burkiewicz and J Kur. 1995. PCR differentiation of seventeen genospecies of *Acinetobacter*. FEMS Microbiol Lett 126: 161–187.
24 Pot B, P Vandamme and K Kersters. 1994. Analysis of electrophoretic whole-organism protein fingerprints. In: Chemical Methods in Prokaryotic Systematics (Goodfellow M and AG O'Donnell, eds), John Wiley and Sons, London.
25 Rainey FA, E Lang and E Stackebrandt. 1994. The phylogenetic structure of the genus *Acinetobacter*. FEMS Microbiol Lett 124: 341–354.
26 Sneath PHA and RR Sokal. 1973. The Principles and Practice of Numerical Classification. WH Freeman, San Francisco.
27 Soddell JA, AM Beacham and RJ Seviour. 1993. Phenotypic identification of non-clinical isolates of *Acinetobacter* species. J Appl Bacteriol 74: 210–214.
28 Tjernberg I and J Ursing. 1989. Clinical strains of *Acinetobacter* classified by DNA–DNA hybridization. Acta Path Microbiol Immunol Scand 97: 595–605.
29 Toerien DF, A Gerber, LH Lotter and TE Cloete. 1990. Enhanced biological phosphorus removal in activated sludge systems. In: Advances in Microbial Ecology (Marshall KC, ed), pp 173–320, Plenum, New York.
30 Vauterin L, J Swing and K Kersters. 1993. Protein electrophoresis and classification. In: Handbook of New Bacterial Systematics (Goodfellow M and AG O'Donnell, eds), pp 251–272, Academic Press, London.
31 Widemann-Al-Ahmad M, H-V Tichy and G Schon. 1994. Characterization of *Acinetobacter* type strains and isolates obtained from wastewater treatment plants by PCR fingerprinting. Appl Environ Microbiol 60: 4066–4071.