Transfer of *Pseudomonas elongata* Humm 1946 to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov.

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Phylogenetic analysis based on 16S rDNA sequences revealed that *Pseudomonas elongata* Humm 1946 is more closely related to the genus *Microbulbifer* than to authentic pseudomonads. The type strain of *P. elongata* (DSM 6810^T) exhibited 16S rDNA similarity levels of 97·5 and 98·2 % to the type strains of *Microbulbifer hydrolyticus* and *Microbulbifer salipaludis*, respectively, but of less than approximately 92 % to *Pseudomonas* species with known 16S rDNA sequences. Respiratory lipoquinone and cellular fatty acid analyses showed that the type strain of *P. elongata* has characteristics similar to those of the genus *Microbulbifer*, not those of the genus *Pseudomonas*. *P. elongata* DSM 6810^T contained ubiquinone-8 as the predominant respiratory lipoquinone and iso-$C_{15:0}$ as the major fatty acid. DNA–DNA relatedness data indicate that *P. elongata* is a species distinct from *M. hydrolyticus* and *M. salipaludis*. Therefore, on the basis of these data, *P. elongata* Humm 1946 should be transferred to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov.

The genus *Pseudomonas* Migula 1894 was defined to accommodate Gram-negative, strictly aerobic rods with polar flagella. This simple classification led to taxonomic heterogeneity in the single genus *Pseudomonas*, which now harbours a large number of species (Skerman *et al.*, 1980; Palleroni, 1984). Over the last two decades, advances in chemical analysis and genomic approaches have played an important role in elucidation of the taxonomic heterogeneity and establishment of reliable systematics in the genus *Pseudomonas* (Oyaizu & Komagata, 1983; Kersters *et al.*, 1996; Anzai *et al.*, 2000). Many species assigned to the genus *Pseudomonas* have been reclassified as members of novel genera or transferred to other genera (Willems *et al.*, 1989, 1990; Yabuuchi *et al.*, 1990, 1992; Urakami *et al.*, 1992; Meyer *et al.*, 1993; Segers *et al.*, 1994; Grimes *et al.*, 1997). Recently, results of 16S rDNA sequence analysis showed that *Pseudomonas elongata* is phylogenetically more closely related to the genus *Microbulbifer* than to the genus *Pseudomonas* (Anzai *et al.*, 2000). De Vos *et al.* (1989) have already reported, on the basis of DNA–rRNA hybridization data, that *P. elongata* was misnamed and is hence not a member of the genus *Pseudomonas*. However, *P. elongata* is slightly different from members of the genus *Microbulbifer* in its colony and cell morphologies. Accordingly, the aim of the present study was to determine the exact taxonomic position of *P. elongata* by using a combination of chemotaxonomic and detailed phylogenetic analyses, based on newly determined 16S rDNA sequence and genomic relatedness. On the basis of the data presented below, we propose that *P. elongata* should be transferred to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov.

P. elongata DSM 6810^T was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Cell biomass of *P. elongata* DSM 6810^T was obtained from marine broth (MB; Difco) cultures at 30 °C for respiratory lipoquinone analysis and DNA extraction. This strain was cultivated on a gyratory shaker at 150 r.p.m. and broth cultures were checked for purity by using a light microscope before harvesting by centrifugation. For fatty acid methyl ester analysis, cell mass of *P. elongata* DSM 6810^T, *Microbulbifer hydrolyticus* DSM 11525^T and *Microbulbifer salipaludis*

The GenBank/EMBL/DDBJ accession number for the newly determined 16S rDNA sequence of *Pseudomonas elongata* DSM 6810^T is AF500006.

SM-1^T was obtained after incubation for 3 days on marine agar (MA). *P. elongata* DSM 6810^T was cultivated at 30 °C and *M. hydrolyticus* DSM 11525^T and *Microbulbifer salipaludis* SM-1^T were cultivated at 37 °C. Chromosomal DNA was isolated and purified according to a previously described method (Yoon *et al.*, 1996), except that ribonuclease T1 was used with ribonuclease A. Respiratory lipoquinones were analysed as described by Komagata & Suzuki (1987) by using reversed-phase HPLC. For quantitative analysis of cellular fatty acid composition, a loop of cell mass was harvested and fatty acid methyl esters were prepared and identified according to the instructions of the Microbial Identification system (MIDI). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. DNA of *P. elongata* DSM 6810^T was used as the labelled DNA probe; reciprocal hybridization was not performed. Of the values obtained, the highest and lowest values for each sample were excluded and the remaining three values were used to calculate similarity values. DNA relatedness values quoted are the means of these three values.

16S rDNA was amplified by PCR with two universal primers, as described previously (Yoon *et al.*, 1998). The PCR product was purified with a QIAquick PCR Purification kit (Qiagen). Sequencing of the purified 16S rDNA PCR product was performed by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) as recommended by the manufacturer. Purified sequencing reaction mixtures were electrophoresed automatically on an Applied Biosystems model 377 DNA sequencer. Alignment of sequences was carried out by using CLUSTAL W software (Thompson *et al.*, 1994). Gaps at the 5′ and 3′ ends of the alignment were omitted from further analysis. Phylogenetic trees were inferred by using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods from the PHYLIP package (Felsenstein, 1993). Evolutionary distance matrices for the neighbour-joining method were calculated by using the algorithm of Jukes & Cantor (1969) with the DNADIST program. Stability of relationships was assessed by bootstrap analysis, based on 1000 resamplings of the neighbour-joining dataset, by using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE of the PHYLIP package.

Colonies of *P. elongata* on MA are yellowish-brown in colour, whereas colonies of *Microbulbifer* species on MA are cream or greyish-yellow (Palleroni, 1984; González *et al.*, 1997). Cells of *P. elongata* are longer than those of *Microbulbifer* species (Palleroni, 1984; González *et al.*, 1997). Despite these slight differences in morphology, *P. elongata* exhibited a much higher phylogenetic affiliation to the genus *Microbulbifer* than to authentic pseudomonads and other genera. The newly determined, almost-complete 16S rDNA sequence of the type strain of *P. elongata* comprised 1491 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Phylogenetic trees based on 16S rDNA sequences show that *P. elongata* does not fall within the cluster that comprises authentic pseudomonads. In the phylogenetic tree based on the neighbour-joining algorithm, *P. elongata* DSM 6810^T forms a coherent cluster with the clade that comprises *Microbulbifer* species, with a bootstrap confidence level of 100 % (Fig. 1). This topology was also found in the trees generated by the maximum-parsimony and maximum-likelihood algorithms. *P. elongata* DSM 6810^T exhibited 16S rDNA similarity levels of 97·5 % to the type strain of *M. hydrolyticus* and 98·2 % to the type strain (SM-1^T) of *M. salipaludis*, which has been recently

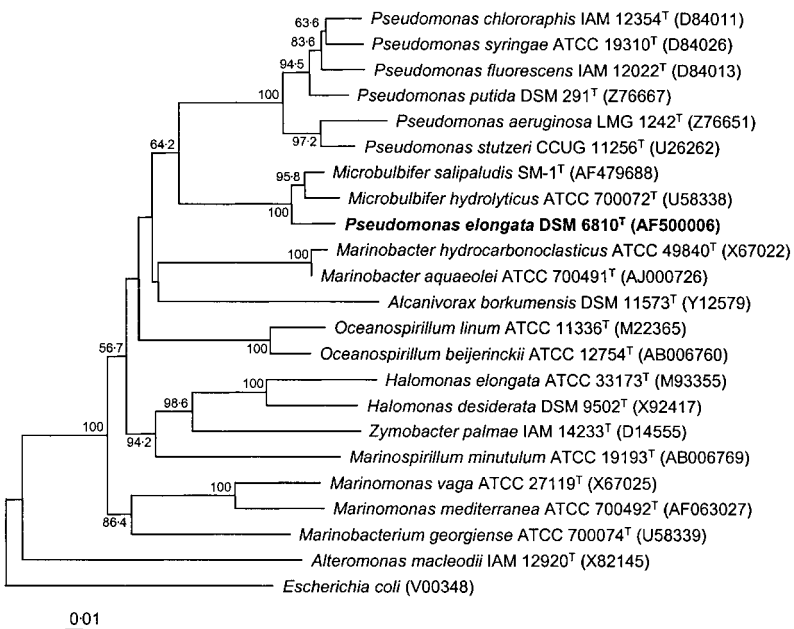

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of *Pseudomonas elongata* and representatives of the γ-subclass of the *Proteobacteria*, based on 16S rDNA sequences. Bootstrap values (percentage of 1000 replications) >50 % are shown at branch-points. Bar, 0·01 substitutions per nucleotide position.

described as the second species of the genus *Microbulbifer* (Yoon *et al.*, 2003). However, 16S rDNA similarity levels between *P. elongata* DSM 6810^T and authentic *Pseudomonas* species with known 16S rDNA sequences were <92 %.

Some chemotaxonomic data also provide supporting evidence that *P. elongata* is more closely related to the genus *Microbulbifer* than to the genus *Pseudomonas*. In this study, the predominant respiratory lipoquinone found in the type strain of *P. elongata* was ubiquinone-8 (Q-8), at a peak ratio of approximately 90 %. Members of the genus *Microbulbifer* were also found to contain Q-8 as the predominant respiratory lipoquinone (Yoon *et al.*, 2003), whereas authentic pseudomonads are known to contain ubiquinone-9 (Oyaizu & Komagata, 1983; Yumoto *et al.*, 2001). In the cellular fatty acid analysis, the type strain of *P. elongata* produced a profile similar to those of *Microbulbifer* species (Table 1); *P. elongata* DSM 6810^T and the type strains of *Microbulbifer* species contained iso-$C_{15:0}$ as the major fatty acid (Table 1). However, authentic pseudomonads have $C_{16:0}$ or $C_{18:1}$ as the major fatty acid (Oyaizu & Komagata, 1983; Yumoto *et al.*, 2001). Therefore, the results obtained in the phylogenetic and chemotaxonomic analyses clearly indicate that *P. elongata* is a member of the genus *Microbulbifer* and not of the genus *Pseudomonas*.

There are some differences in morphological and physiological properties between *P. elongata* and two *Microbulbifer* species (Palleroni, 1984; González *et al.*, 1997; Yoon *et al.*, 2003) (Table 2). DNA–DNA hybridization was performed to determine the genomic relatedness between the type strain of *P. elongata* and the type strains of *M. hydrolyticus* and *M. salipaludis*. *P. elongata* DSM 6810^T exhibited DNA–DNA relatedness levels of 11·4 and 8·5 % with *M. hydrolyticus* DSM 11525^T and *M. salipaludis* SM-1^T, respectively. These data clearly indicate that *P. elongata* is a genomic species that is separate from two *Microbulbifer* species (Wayne *et al.*, 1987). Therefore, on the basis of the data presented here, it is proposed that *P. elongata* Humm 1946 should be transferred to the genus *Microbulbifer* as *Microbulbifer elongatus* comb. nov.

Table 1. Cellular fatty acid profiles of *Microbulbifer hydrolyticus* DSM 11525^T, *Microbulbifer salipaludis* SM-1^T and *Pseudomonas elongata* DSM 6810^T

Species: 1, *M. hydrolyticus* DSM 11525^T; 2, *M. salipaludis* SM-1^T; 3, *P. elongata* DSM 6810^T. Values are percentages of total fatty acids. −, Not detected. Fatty acids that represent <0·5 % are omitted.

Fatty acid	1	2	3
Saturated:			
$C_{10:0}$	1·7	2·4	1·6
$C_{10:0}$ 3-OH	1·0	1·2	1·6
$C_{14:0}$	1·2	2·6	0·7
$C_{15:0}$	1·5	1·7	0·9
$C_{16:0}$	11·4	16·3	7·1
$C_{16:0}$ 2-OH	−	0·9	−
$C_{17:0}$	2·9	2·2	2·5
$C_{17:0}$ cyclo	5·7	−	−
$C_{18:0}$	1·6	1·4	1·2
$C_{19:0}$ cyclo $\omega 8c$	1·0	−	−
Unsaturated:			
$C_{17:1}\omega 8c$	0·5	1·0	1·8
$C_{18:1}\omega 5c$	−	0·7	−
$C_{18:1}\omega 7c$	8·9	11·8	16·3
Branched:			
iso-$C_{11:0}$	5·7	4·8	6·5
iso-$C_{11:0}$ 3-OH	6·2	5·7	7·7
iso-$C_{15:0}$	24·4	19·4	20·7
iso-$C_{15:1}$ F*	1·0	0·7	1·0
iso-$C_{16:0}$	−	−	0·5
iso-$C_{17:0}$	10·4	5·5	9·9
iso-$C_{17:0}$ 3-OH	−	0·9	−
anteiso-$C_{17:0}$	−	−	0·8
iso-$C_{17:1}\omega 9c$	10·1	9·5	11·3
Summed feature†:			
3	2·7	7·1	6·0
4	−	1·7	−

*The double bond position indicated by a capital letter is unknown. †Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 contained the following fatty acids: iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega 7c$. Summed feature 4 contained the following fatty acids: anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I.

Description of *Microbulbifer elongatus* (Humm 1946) comb. nov.

Microbulbifer elongatus (e.lon.ga′tus. L. masc. part. adj. *elongatus* elongated, stretched out).

The description is as that given by Humm (1946) and Palleroni (1984). Some characteristics are as follows. Cells are slender, single and occur in chains or filaments of indefinite length; rod-shaped with dimensions of 0·3–0·4 × 3·0–6·0 μm. Longer and coccoid cells are also present. Aerobic, Gram-negative, motile, non-spore-forming and encapsulated. Colonies in agar are sunken due to agar liquefaction. Yellowish-brown pigment that diffuses into the medium is produced from peptone. Optimal growth temperature is 25–30 °C. Salts are required for growth; optimal salt concentration for growth is 2–3 % (w/v). Catalase-positive. Urease-negative. Alginic acid, cellulose, chitin and starch are hydrolysed. Gelatin is liquefied slowly. Negative for nitrate reduction. Hydrogen sulfide is produced, but indole is not. Acid is produced from arabinose, cellobiose, maltose, salicin, sucrose and xylose. Ammonia, nitrate, nitrite or amino acids can be used as nitrogen sources. Fructose, galactose, glucose, mannose, lactose, acetate, lactate, malate, propionate, succinate, D-arginine, glutamate and L-leucine are utilized. DL-Alanine, aspartate, cystine, glycine, L-proline (poorly) and tyrosine (poorly) are

Table 2. Differential phenotypic characteristics of *Microbulbifer hydrolyticus*, *Microbulbifer salipaludis* and *Pseudomonas elongata*

+, Positive reaction; −, negative reaction; ND, not determined. All species were positive for hydrolysis of starch and catalase and production of acid from D-cellobiose and maltose. All species were negative for spore formation, Gram staining, acid production from D-fructose, D-galactose, lactose and D-mannose and growth in 0 % NaCl.

Characteristic	*M. hydrolyticus**	*M. salipaludis*†	*P. elongata*‡
Cell morphology	Rods	Rods	Cocci or rods
Colour of colonies	Cream	Greyish-yellow	Yellowish-brown
Nitrate reduction to nitrite	ND	ND	−
Decomposition of:			
Cellulose	+	ND	+
Chitin	+	−	+
Gelatin	+	ND	+
Acid production from:			
L-Arabinose	+	−	+
D-Glucose	+	+	−
Sucrose	−	−	+
D-Xylose	+	−	+
Optimum growth temperature (°C)	37	37	25–30
Predominant ubiquinone	Q-8†	Q-8	Q-8§
Major fatty acid(s)	iso-$C_{15:0}$	iso-$C_{15:0}$	iso-$C_{15:0}$§
DNA G+C content (mol%)	58	59	ND

*Data from González *et al.* (1997).

†Data from Yoon *et al.* (2003).

‡Data from Humm (1946) and Palleroni (1984).

§Data from this study.

utilized only as nitrogen sources. Rhamnose, raffinose, inulin, ethanol, ethylene glycol, glycerol, mannitol, dulcitol, inositol, sorbitol, creatine, L-methionine, DL-phenylalanine, DL-serine, DL-valine, butyrate, citrate, gluconate, maleate, malate, malonate, mucate, oxalate, tartrate and iso-valerate are not utilized. Further descriptive information is given by Humm (1946). Additional characters found in this study are as follows: predominant respiratory lipoquinone is ubiquinone-8 (Q-8). Major fatty acid is iso-$C_{15:0}$ and significant amounts of $C_{18:1}\omega7c$ and iso-$C_{17:1}\omega9c$ also exist.

Isolated from intertidal sand, sea water and bottom sediments. The type strain is ATCC 10144^T (=DSM 6810^T=LMG 2182^T).

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