

Chitinibacter tainanensis gen. nov., sp. nov., a chitin-degrading aerobe from soil in Taiwan

Lih-Ling Chern,¹ Erko Stackebrandt,² Shu-Fen Lee,¹ Fwu-Ling Lee,¹
Jeen-Kuan Chen³ and Huei-Mei Fu¹

Correspondence
Fwu-Ling Lee
fill@firdi.org.tw

¹Bioresource Collection and Research Centre, Food Industry Research and Development Institute, PO Box 246, Hsinchu 300, Taiwan

²DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b, D-38124 Braunschweig, Germany

³Department of Environment and Biotechnology, Refining and Manufacturing Research Centre, Chinese Petroleum Corporation, Chia-Yi, Taiwan

Five strains with strong chitinolytic activity were isolated from a soil sample collected from southern Taiwan. The strains shared more than 92% DNA–DNA similarity, indicating membership of the same genospecies. This close relationship was supported by high similarities in fatty acid composition and biochemical characteristics. A 16S rRNA gene sequence analysis indicated that the isolates were members of the class ‘*Betaproteobacteria*’, in which they formed an individual subline of descent that was distantly related (<94% similarity) to lineages defined by *Formivibrio citricus* DSM 6150^T and *Iodobacter fluviatilis* DSM 3764^T. On the basis of the phylogenetic and phenotypic distinctness of these novel chitin-degrading organisms, a new genus, *Chitinibacter*, is proposed, with *Chitinibacter tainanensis* (type strain, S1^T=BCRC 17254^T=DSM 15459^T) as the type species.

Next to cellulose, chitin is the second most-abundant polysaccharide on Earth, as it is a major component of most fungal cell walls, insect exoskeletons and the shells of crustaceans (Flach *et al.*, 1992). Derivatives of chitin, including polysaccharides, oligosaccharides and monosaccharides, have been shown to play a role in the organogenesis of plants and the embryogenesis of invertebrates (Bakkers *et al.*, 1997). Recently, chitin oligosaccharides have been demonstrated to enhance functions of the immunological system in host animals, possibly functioning as tumoricidal agents (Suzuki *et al.*, 1986). To prepare functional chitin derivatives, micro-organisms were isolated from soils and screened for chitinase activities. This report deals with the detailed characterization of five strains of bacteria with strong chitinolytic activity and with the proposal of *Chitinibacter tainanensis* gen. nov., sp. nov.

Chitinolytic bacteria were isolated from various soil samples collected from Tainan Prefecture in the southern part of Taiwan, using agar medium containing 0.2% colloidal chitin (Hsu & Lockwood, 1975) as a selective medium. The

reduced sugars formed in the medium, indicating the degradation of chitin, were detected according to a modified procedure of Imoto & Yagishita (1971). Colonies with surrounding clear zones were selected and incubated at 30 °C in Luria–Bertani (LB) medium (Life Science).

Micro-organisms used for comparison with the isolated chitin-degrading strains were *Alcaligenes latus* ATCC 29712^T [obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA], *Iodobacter fluviatilis* DSM 3764^T and *Formivibrio citricus* DSM 6150^T [both obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany]. Growth media for *I. fluviatilis* and *A. latus* were nutrient agar (Difco) and brain–heart infusion medium (Difco), while *F. citricus* was cultivated on *Formivibrio* medium (medium DSM 505; DSMZ, 2001). Cultivation was at 30 °C. Ten strains of chitin-degrading micro-organisms were isolated from soil samples collected in the southern part of Taiwan. Strains isolated from Tainan grew at 30 °C aerobically with shaking, and showed the strongest chitin-degrading potential, as indicated by determination of the reducing power in the medium. Strain S1^T, which was deposited at the Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Taiwan as *C. tainanensis* (as BCRC 17254^T) and at the DSMZ (as DSM 15459^T), and strains S5, S6, S9 and S12 were selected for further studies.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Chitinibacter tainanensis* S1^T is AY264287.

The phenotypic characteristics (Table A) and cellular fatty acid compositions (Table B) of the novel chitin-degrading strains, and DNA–DNA hybridization results (Table C), are available as supplementary data in IJSEM Online.

All tests on the novel bacteria were performed using LB medium at 30 °C unless stated otherwise. The cell morphology of the strains was observed using a Zeiss light microscope. To visualize flagella, a drop of bacterial suspension was gently transferred to a copper grid (square 100-mesh) which was cleaned with acetone, coated with colloidal, dried in a desiccator overnight and then stabilized with carbon in a high-vacuum coating unit (HUS-5GB; Hitachi). Excess water was removed by absorption with filter paper from the side of the grid. Bacteria retained on the grid were negatively stained with 1% phosphotungstic acid (pH 7) for 60 s. After the stain solution had been removed from the grid with filter paper, the images were viewed and photographed with a Hitachi H-600 transmission electron microscope. A Gram stain was performed using the Gram-colour set (Merck). Growth at various temperatures, ranging from 5 to 50 °C, using 5 °C increments, was determined on LB medium. The diameters of colonies were measured after incubation at 30 °C for 3 days. The effect of pH on growth was tested with 0.5 × LB medium buffered with 0.2 M citric acid and Na₂HPO₄ and adjusted to pH values 3, 4 and 5–10 (using increments of 0.5) with NaOH or HCl. Aliquots (5 ml) of the pH-buffered medium in 13 × 100 mm screw-capped test tubes were inoculated with 0.25 ml bacterial suspension (concentration equal to McFarland 1) prepared from a 2-day-old culture. Four replicates were used for each pH test. After incubation under shaking for 7 days, growth was indicated by a change in optical density at 400 nm, measured using a spectrophotometer (Walden Precision Apparatus). The pH was stable during the incubation. The oxygen requirement was tested by using growth distribution following a shake-tube inoculation method (Cappuccino & Sherman, 1987). The presence of cytochrome oxidase was tested using oxidase reagent droppers (Difco); catalase activity was determined using H₂O₂ solution as described by Koneman *et al.* (1979). The abilities of the strains to utilize various substrates as sole carbon sources were evaluated using the API 20NE panel (bioMérieux) and Vitek NFC cards (bioMérieux) according to the manufacturer's instructions.

Hydrolysis of chitin by the isolated strains was tested using agar medium supplemented with colloidal chitin as described above. *Cytophaga* sp. DSM 3657 and *Serratia marcescens* DSM 30121^T were used as chitinase-positive control strains. *I. fluviatilis* DSM 3764^T and *F. citricus* DSM 6150^T were also tested, as they were phylogenetic neighbours of the novel isolates. The preparation of colloidal chitin was according to the description of Monreal & Reese (1969). The agar medium contained the following (l⁻¹): 0.3 g MgSO₄·7H₂O, 1.36 g KH₂PO₄, 1.0 g (NH₄)₂SO₄, 1.0 g NaCl, 4.0 g colloidal chitin, 1.0 g yeast extract, 12.0 g agar, 5.0 ml trace elements (DSMZ medium 27; DSMZ, 2001) and vitamin solution (DSMZ medium 428; DSMZ, 2001). For growth of strain DSM 6150^T, DSMZ medium 505 was used in which malate was replaced by 1.5 g colloidal chitin. Tests for chitinase activity were done under aerobic

conditions, except in the case of *F. citricus* DSM 6150^T, which was tested under an N₂ atmosphere.

All isolates were strictly aerobic, oxidase-positive and catalase-positive. All strains showed almost identical reactions in the API and Vitek substrate-utilization panels. Results are included in the species description, and the data are available in IJSEM Online (Table A). All of the novel strains grew on LB medium and on trypticase–soy agar plus blood at temperatures ranging from 10 to 40 °C, with an optimum temperature between 30 and 37 °C. No growth was observed at 5 or 45 °C after incubation for 14 days. Growth of the novel bacteria occurred at pH 5.5–9.0, as indicated by the increase in optical density at 400 nm. The optimum pH was around pH 7.5.

While the chitinase-positive reference strains *Cytophaga* sp. DSM 3657 and *S. marcescens* DSM 30121^T, as well as the novel isolate S1^T, were strongly positive, the reaction of *I. fluviatilis* DSM 3764^T was very weak and delayed, producing a small clear zone of about 2 mm around the large colonies of 2.5 cm in diameter. *F. citricus* DSM 6150^T did not grow at all on the chitin agar, while the control grew on the same medium in which chitin had been replaced by trisodium acetate. This strain should therefore be considered chitin-negative.

The ubiquinone system of the novel chitin-degrading bacteria was determined by using the method described by Collins & Jones (1981). The TLC-purified quinones were analysed with a Nova-Pak C₁₈ (15 × 3.9 cm) column (Waters). Peaks were detected at 275 nm after elution with methanol/2-propanol (2:1) at flow rate of 1 ml min⁻¹. Q-6, Q-7, Q-9 and Q-10 standards were purchased from Sigma. Q-8 was extracted from the *A. latus* ATCC 29712^T culture. Q-8 was found to be the major quinone in strain S1^T (98% of the total quinones) and *I. fluviatilis* DSM 3764^T (95%); this is quite common among members of the 'Betaproteobacteria'.

Cellular fatty acid compositions of the novel isolates and *I. fluviatilis* DSM 3764^T and *F. citricus* DSM 6150^T were determined using the Sherlock Microbial Identification System (MIDI). Extracts of the methylated fatty acids were prepared according to the protocol provided by the manufacturer and analysed with a 5890 gas chromatograph equipped with a flame ionization detector and an automatic injector with a G1512A controller (Hewlett Packard). Identification of the peaks was made by comparing the results with the built-in TSBA 40 database (MIDI). The major fatty acids (>1% of total fatty acids) of the chitin-degrading strains were as follows: 16:1 ω 7c/15 iso 2-OH (48.45–51.83%), 18:1 ω 7c (17.72–19.85%), 16:0 (15.36–17.57%), 12:0 3-OH (3.87–4.21%), 12:0 (2.45–3.09%), 10-methyl 19:0 (1.37–2.03%) and 16:0 3-OH (1.24–1.44%). A complete listing of the fatty acids is available in IJSEM Online (Table B). The differences in fatty acid profiles of phylogenetically related species are listed in Table 1.

Table 1. Differential characteristics of *C. tainanensis* from phylogenetically related species

Characteristic	<i>C. tainanensis</i> S1 ^T	<i>F. citricus</i> DSM 6150 ^{T*}	<i>I. fluviatilis</i> NCTC 11159 ^{T†}
Anaerobic growth	–	+	–
Chitin hydrolysis	+	–	Weak (delayed)
Cellular fatty acids‡			
12:0 3-OH	3.87–4.21 %	–/Trace	2–3 %
14:0	Trace	2.2 %	8.4 %
14:0 3-OH	–	9.5 %	–
10-methyl 19:0	1.37–2.03 %	–	–
Growth at 40 °C	+	–	–
G + C content (mol%)	55.9–56.2	61	50–52
Major quinone	Q-8	ND	Q-8

ND, Not determined.

*Data for *F. citricus* were taken from Logan (1989), except for chitin hydrolysis and cellular fatty acids.

†Data for *I. fluviatilis* were taken from Tanaka *et al.* (1991) and Hippe *et al.* (1999), except for chitin hydrolysis and cellular fatty acids.

‡All five strains of *C. tainanensis* were tested.

Isolation of genomic DNA for sequence analysis was done as described by Franke *et al.* (1999). PCR amplification of the 16S rRNA gene and the subsequent cycle sequencing reaction were carried out using the MicroSeq 16S rRNA gene kit (Applied Biosystems). The products were analysed with a PRISM 310 genetic analyser (Applied Biosystems). Phylogenetic analyses was performed by using different treeing algorithms [distance matrix (De Soete, 1983; Felsenstein, 1993); neighbour joining and maximum parsimony (Felsenstein, 1993)].

Analysis of the 16S rRNA gene sequence of strain S1^T, consisting of 1514 nt, revealed its closest phylogenetic neighbours to be *F. citricus* DSM 6150^T (Y17602) and *I. fluviatilis* ATCC 33051^T (M22511), members of the ‘Betaproteobacteria’, with similarity values of 93.4% and 90.6%, respectively. Partial 16S rRNA gene sequence analysis (positions 13–518 according to the *Escherichia coli* numbering system; Brosius *et al.*, 1978) of the other four chitin-degrading isolates revealed 100% identity with the homologous stretch of strain S1^T. Different treeing algorithms gave consistent results by placing the chitin-degrader as a new lineage between the *Formivibrio* and *Iodobacter* lineages (Fig. 1). The order of branching points within this cluster is not stable as judged from the bootstrap value of 64%.

A commercial kit (Genomic-tips; Qiagen) was used for the preparation of genomic DNA for determination of the base composition of the DNA (mol% G + C content) and DNA–DNA reassociation. The G + C content was determined by using reversed-phase HPLC (Tamaoka & Komagata, 1984) with slight modifications. The nucleotides were separated using a Cosmosil 5C₁₈ column (4.0 × 150 mm) (Waters) in a mobile phase composed of 0.2 M NH₄H₂PO₄/acetonitrile (20 : 1, v/v) at a flow rate of

1 ml min⁻¹ at room temperature. The nucleotides were detected and quantified by absorption at 270 nm. DNA similarity values between the chitin-degrading strains, and between two of these strains and the phylogenetically nearest type strains of other species, were determined using the fluorometric hybridization method in microdilution wells (Ezaki *et al.*, 1989). The fluorescence intensity of each well was measured with a Fluoroskan II microplate fluorometer (Labsystems) at a wavelength of 360 nm for excitation and at 450 nm for emission. The chitin-degrading

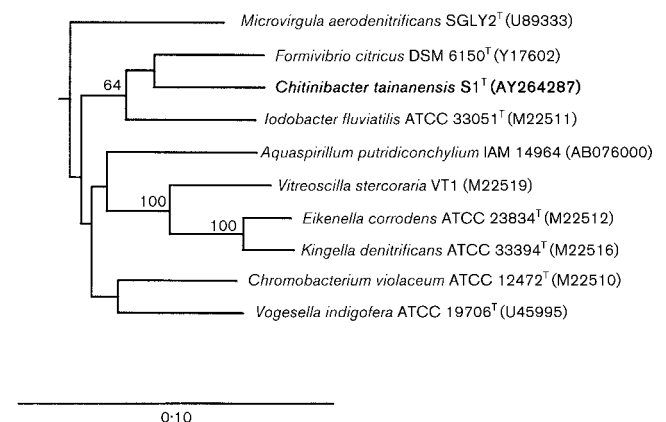


Fig. 1. Phylogenetic tree, based on 16S rRNA gene sequences, showing the nearest neighbours of the novel chitin-degrading bacterium in the class ‘Betaproteobacteria’. The bar corresponds to a 10% difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting any two organisms. The numbers shown at the branch points indicate percentage bootstrap values from 1000 datasets. Only bootstrap values greater than 60% are shown.

strains had a DNA G+C content ranging from 55.9 to 56.2 mol%. This value is significantly lower than the 61 mol% determined for *F. citricus* DSM 6150^T (Hippe *et al.*, 1999) and higher than the 50–52 mol% of *I. fluviatilis* NCTC 11159^T (Logan, 1989). The DNA similarity test revealed that the five strains shared high DNA similarity values (92–100%), indicating membership of the same genospecies. DNA similarity values between strain S1^T or strain S12 and *F. citricus* DSM 6150^T and *I. fluviatilis* DSM 3764^T were below 10%.

The five isolates from Taiwanese soil were identical with respect to morphology, physiology, chemotaxonomy and genomic properties, and should be considered members of the same taxon. Phylogenetically, they form a novel line of descent within the 'Betaproteobacteria', with *Formivibrio* and *Iodobacter* as the closest (albeit remote) neighbours. Strain S1^T and the four similar strains (S5, S6, S9 and S12) can be clearly distinguished from the two phylogenetic neighbours which form two clearly separate genomic entities, as judged from the low sequence-similarity values. The most salient property is the degradation of chitin, a characteristic that is weak or absent in the type strains of the phylogenetic neighbours. Other characteristics separating the chitinolytic taxon from *Formivibrio* and *Iodobacter* are listed in Table 1. Although some chitinolytic bacteria have been shown to be insect pathogens (Sampson & Gooday, 1998), the question of whether these new Taiwanese strains are pathogenic to insects remains to be investigated.

Description of the genus *Chitinibacter* gen. nov.

Chitinibacter (chi.ti.ni.bac'ter. N.L. n. *chitinum* chitin; N.L. masc. n. *bacter* rod; N.L. masc. n. *Chitinibacter* rod that degrades chitin).

Strictly aerobic, Gram-negative rods that are straight to slightly curved (Fig. 2a) with round ends, 1.3–2.6 µm in length and 0.5–0.9 µm in width. Highly motile by means of one polar flagellum or two polar flagella (Fig. 2b, c). Oxidase- and catalase-positive. No acid from glucose. Q-8 is the predominant isoprenoid quinone. Major fatty acids (>5%) are 16:1 ω 7c/15 iso 2-OH (48.45–51.83%), 18:1 ω 7c (17.72–19.85%) and 16:0 (15.36–17.57%). Phylogenetically, a member of the class 'Betaproteobacteria'. The G+C content of the DNA is 56 mol%.

The type species is *Chitinibacter tainanensis*.

Description of *Chitinibacter tainanensis* sp. nov.

Chitinibacter tainanensis (tai.na.nen'sis. N.L. adj. *tainanensis* referring to Tainan, a town in Southern Taiwan, the origin of the soil sample from which the type strain was isolated).

In addition to the properties defining the genus, strains of the species form milky white, circular, entire, convex colonies on LB agar, measuring 0.5–2.0 mm in diameter. Growth occurs at temperatures from 10 to 40 °C, with the

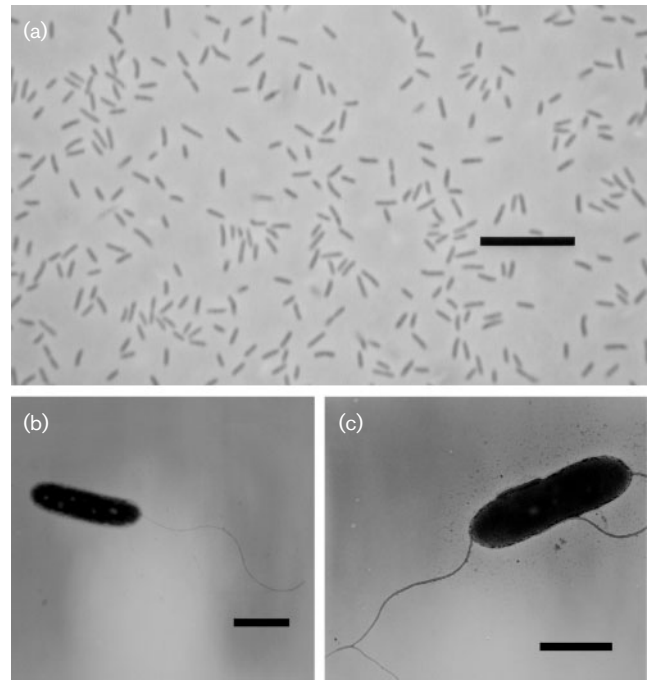


Fig. 2. Phase-contrast photomicrograph (a) and negatively stained electron micrographs (b, c) of *C. tainanensis* cells, showing one polar flagellum or two polar flagella. Bars: 10 µm (a); 1 µm (b, c).

optimum at 30–37 °C, and at pH values from 5.5 to 9.0, with the optimum at pH 7.5. Negative for nitrate reduction, indole production, arginine dihydrolase, urease, β -glucosidase, β -galactosidase and protease. On the basis of API and Vitek tests, glucose, *N*-acetylglucosamine and gluconate are utilized. The following are not utilized: arabinose and D-mannose (strain S6 gave a weak reaction for both in API tests), D-mannitol, maltose and citrate. API tests were negative for maltose, caprate, adipate, malate and phenyl acetate. Vitek tests were negative for *myo*-inositol, D-galactose, sucrose, trehalose, D-xylose, DL-lactate, sodium acetic acid, propionic acid, adipic acid, itaconic acid, suberic acid, sebacic acid, azelaic acid, heptanoic acid, L-aspartic acid, L-alanine, L-histidine and L-proline. Isolated from Taiwan, near Tainan Prefecture (120° 16' 21" E 23° 13' 09" N).

Type strain is S1^T (= BCRC 17254^T = DSM 15459^T).

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References

- Bakkers, J., Semino, C. E., Stroband, H., Kijne, J. W., Robbins, P. W. & Spaik, H. P. (1997). An important developmental role for oligosaccharides during early embryogenesis of cyprinid fish. *Proc Natl Acad Sci U S A* **94**, 7982–7986.
- Brosius, J., Palmer, M. L., Kennedy, P. J. & Noller, H. F. (1978). Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci U S A* **75**, 4801–4805.
- Cappuccino, J. G. & Sherman, N. (1987). *Microbiology: a Laboratory Manual*. Menlo Park, CA: Benjamin/Cummings Publishing.
- DSMZ (2001). *Catalogue of Strains*, 7th edn. Braunschweig: Deutsche Sammlung von Mikroorganismen und Zellkulturen.
- Collins, M. D. & Jones, D. (1981). A note on the separation of natural mixtures of bacterial ubiquinones using reverse-phase partition thin-layer chromatography and high performance liquid chromatography. *J Appl Bacteriol* **51**, 129–134.
- DeSoete, G. (1983). A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**, 621–626.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1993). PHYLIP (phylogeny inference package), version 3.5c. Department of Genetics, University of Washington, Seattle, USA.
- Flach, J., Pilet, P. E. & Jolles, P. (1992). What's new in chitinase research? *Experientia* **48**, 701–716.
- Franke, I. H., Fegan, M., Hayward, C., Leonard, G., Stackebrandt, E. & Sly, L. I. (1999). Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug. *Int J Syst Bacteriol* **49**, 1681–1693.
- Hippe, H., Hagelstein, A., Kramer, I., Swiderski, J. & Stackebrandt, E. (1999). Phylogenetic analysis of *Formivibrio citricus*, *Propionivibrio dicarboxylicus*, *Anaerobiospirillum thomasi*, *Succinimonas amylolytica* and *Succinivibrio dextrinosolvans* and proposal of *Succinivibrionaceae* fam. nov. *Int J Syst Bacteriol* **49**, 779–782.
- Hsu, S. C. & Lockwood, J. L. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Appl Microbiol* **29**, 422–426.
- Imoto, T. & Yagishita, K. (1971). A simple activity measurement of lysozyme. *Agric Biol Chem* **35**, 1154–1156.
- Koneman, E., Allen, S., Dowell, V. & Sommers, H. (1979). *Color Atlas and Textbook of Diagnostic Microbiology*, pp. 216–217. Philadelphia: J. B. Lippincott.
- Logan, N. A. (1989). Numerical taxonomy of violet-pigmented, Gram-negative bacteria and description of *Iodobacter fluviatile* gen. nov., comb. nov. *Int J Syst Bacteriol* **39**, 450–456.
- Monreal, J. & Reese, E. T. (1969). The chitinase of *Serratia marcescens*. *Can J Microbiol* **15**, 689–696.
- Sampson, M. N. & Gooday, G. W. (1998). Involvement of chitinases of *Bacillus thuringiensis* during pathogenesis in insects. *Microbiology* **144**, 2189–2194.
- Suzuki, K., Mikami, T., Okawa, Y., Tokoro, A., Suzuki, S. & Suzuki, M. (1986). Antitumor effect of hexa-*N*-acetylchitohexaose and chitohexaose. *Carbohydr Res* **151**, 403–408.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Tanaka, K., Nakamura, K. & Mikami, E. (1991). Fermentation of S-citramalate, citrate, mesaconate, and pyruvate by a Gram-negative strictly anaerobic non-spore-former, *Formivibrio citricus* gen. nov., sp. nov. *Arch Microbiol* **155**, 491–495.