Halalkalibacillus halophilus gen. nov., sp. nov., a novel moderately halophilic and alkaliphilic bacterium isolated from a non-saline soil sample in Japan

Akinobu Echigo,^{1,2} Tadamasa Fukushima,^{1,2} Toru Mizuki,^{1,2} Masahiro Kamekura³ and Ron Usami^{1,2}

Correspondence

Akinobu Echigo

dc0400017@toyonet.toyo.ac.jp

¹Department of Applied Chemistry, Faculty of Engineering, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

²Bio-Nano Electronics Research Centre, Toyo University, 2100 Kujirai, Kawagoe, Saitama 350-8585, Japan

³Noda Institute for Scientific Research, 399 Noda, Noda, Chiba 278-0037, Japan

A moderately halophilic and alkaliphilic bacterium, designated strain BH2^T, was isolated from non-saline garden soil in Saitama, Japan. Cells of strain BH2^T were motile, aerobic, rod-shaped and Gram-positive and contained A1 γ , meso-diaminopimelic acid-type murein. Growth occurred in 5.0–25 % (w/v) NaCl (optimum, 10–15 %, w/v), at pH 5.5–10.0 (optimum, pH 8.5–9.0) and at 20–40 °C. The predominant isoprenoid quinone was menaquinone-7. The major cellular fatty acids were ai-C_{15:0}, i-C_{16:0}, ai-C_{17:0} and i-C_{15:0}. The G + C content of the total DNA of strain BH2^T was 35.1 $\pm$ 0.4 mol% ($\pm$ SD; n = 5). The phylogenetic distance from species with validly published names was less than 94.1 %. The phylogenetic and phenotypic characteristics indicated that strain BH2^T represents a novel genus and species, for which the name *Halalkalibacillus halophilus* gen. nov., sp. nov. is proposed. The type strain is BH2^T (= JCM 14192^T = DSM 18494^T).

Halophilic micro-organisms are adapted to conditions of high salinity and require a certain concentration of NaCl for optimum growth (Kushner & Kamekura, 1988; Oren, 2002). Moderate halophiles are defined as those that grow optimally in media containing 3–15 % (w/v) NaCl. They have been isolated from various saline environments such as salt lakes (e.g. the Dead Sea and the Great Salt Lake), salterns, solar salts and subsurface salt formation. It is tacitly believed that the habitats of moderate halophiles are restricted to saline environments and no reports have been published on the isolation of moderately halophilic micro-organisms from non-saline environments. In this study, we propose that a moderately halophilic and alkaliphilic bacterium isolated from a sample from ordinary non-saline garden soil represents a novel genus and species, based on phylogenetic and phenotypic characteristics.

Abbreviation: *m*-Dpm, meso-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BH2^T is AB264529.

An alignment of 16S rRNA gene sequences of members of the family *Bacillaceae* and a phylogenetic tree reconstructed from the alignment and signature bases of the genera *Alkalibacillus*, *Filobacillus*, *Tenuibacillus* and strain BH2^T are available as supplementary material in IJSEM Online.

Soil samples were taken from non-saline, ordinary gardens in many cities of Saitama Prefecture, Japan. Most of the gardens were full of trees and flowers. No highly saline environments such as salterns or salt lakes occur in the region. NaCl contents calculated by determining the Cl[−] content of a soil extract with water using the method of Mohr (Lenore *et al.*, 1998) were less than 0.1 % (w/v). The pH values of the soil extracts were slightly acidic, pH 5.0–6.0.

About 0.5 g of the soil sample was placed on an alkaline agar plate, spread with a spatula and incubated at 37 °C for 3 weeks in a plastic bag to prevent desiccation. The medium used contained the following (l^{−1}): 5.0 g Casamino acids (Difco), 5.0 g yeast extract (Difco), 1.0 g sodium glutamate.H₂O, 3.0 g trisodium citrate.2H₂O, 2.0 g KCl, 0.2 g MgSO₄.7H₂O, 36 mg FeCl₂.4H₂O, 200.0 g (3.4 M) NaCl and 20 g Bacto agar (Difco), pH 7.2. After autoclaving, the pH was adjusted to 9.5 by addition of pre-calculated amounts of sterile Na₂CO₃ solutions. Colonies were picked, transferred to fresh agar plates, and pure cultures were obtained by plating serial dilutions and repeated transfers on agar plates. Strain BH2^T, isolated from non-saline surface soil from a garden in Niiza City, was selected for further characterization after sequencing of PCR-amplified 16S rRNA genes of a few isolates.

Strain BH2^T grew in 5.0–25 % (w/v) NaCl (pH 9.5, 37 °C), with optimum growth at 10–15 % (w/v) NaCl. Growth occurred within the pH range 5.5–10.0 [10 % (w/v) NaCl, 37 °C], with optimum growth at pH 8.5–9.0. The temperature range for growth was 20–40 °C [10 % (w/v) NaCl, pH 9.5], with optimum growth at 30–37 °C. Anaerobic growth was not observed using an anaerobic jar (Echigo *et al.*, 2005) after incubation for 7 days at 37 °C.

Colonies of strain BH2^T formed on agar (10 % NaCl, pH 9.5, 37 °C) were cream-like and opaque. The cells were rod-shaped and approximately 0.3–0.5 × 2.0–3.0 µm. The cells were motile with a single polar flagellum, observed according to Kodaka *et al.* (1982). Endospore formation was examined after spore-staining, according to the method of Wirtz-Conklin (Murray *et al.*, 1999). A spherical terminal endospore with a swollen sporangium was produced (at 10 % NaCl, pH 9.5, 37 °C). Cells stained Gram-positive after acetic acid fixation, as described by Dussault (1955). The KOH test (Gregersen, 1978) and L-alanine aminopeptidase using Bactident Aminopeptidase test strips (Merck) were negative, as reported previously for Gram-positive bacteria (Gregersen, 1978).

Production of acid from carbohydrates was tested using media in which the amounts of Casamino acids and yeast extract were reduced to 1.0 g l⁻¹, supplemented with 10.0 g l⁻¹ of the test carbohydrate. The cultures were incubated at 37 °C under aerobic conditions for 3 days. Growth was determined visually and the pH was measured with a pH electrode. Strain BH2^T did not produce acid from D-galactose, maltose, sucrose, D-trehalose, D-mannitol, D-fructose, D-glucose or D-xylose. Tests for catalase and oxidase activities and hydrolysis of starch, gelatin, casein, DNA, hippurate, aesculin, pullulan and Tween 80 were performed according to the standard or modified procedures of Smibert & Krieg (1994), Oren *et al.* (1997) and Schlesner *et al.* (2001). Strain BH2^T was catalase- and oxidase-positive and did not hydrolyse starch, casein, gelatin, DNA, hippurate, aesculin, pullulan or Tween 80. Reduction of nitrate was not detected using the sulfanilic acid and α -naphthylamine reagent (Smibert & Krieg, 1981) and formation of gas from nitrate was not detected using Durham tubes under anaerobic conditions.

The sensitivity to antimicrobial agents was tested with discs impregnated with the particular antimicrobial agents placed on pre-inoculated plates. Inhibitory zones around the discs were recorded after incubation for 3 days at 37 °C. Strain BH2^T was sensitive to ampicillin (50 µg disc⁻¹), bacitracin (25 µg disc⁻¹), chloramphenicol (25 µg disc⁻¹) and novobiocin (25 µg disc⁻¹), whereas it was resistant to kanamycin (50 µg disc⁻¹), streptomycin (100 µg disc⁻¹), tetracycline (50 µg disc⁻¹) and anisomycin (50 µg disc⁻¹).

HPLC analysis of isoprenoid quinones and GC/MS analysis of fatty acid methyl esters were performed according to the modified procedures of Tamaoka (1986) and Komagata & Suzuki (1987). The predominant isoprenoid quinone of

strain BH2^T was menaquinone-7 (MK-7). The cellular fatty acid profile of strain BH2^T was characterized by saturated branched fatty acids such as ai-C_{15:0}, i-C_{16:0}, ai-C_{17:0} and i-C_{15:0}.

Preparation of peptidoglycan and determination of its structure were performed according to the modified procedures of Schleifer & Kandler (1972), Schleifer (1985) and Schlesner *et al.* (2001). Purified peptidoglycan was hydrolysed in 4 M HCl at 100 °C for 16 h (total hydrolysate) or 45 min (partial hydrolysate). Diamino acids were identified from the total hydrolysate by using one-dimensional TLC in methanol/pyridine/4 M HCl/water (80:10:4:26, by vol.) solvent system. Amino acids and peptides were identified from the partial hydrolysate by using two-dimensional TLC. The first direction was developed in isopropyl alcohol/acetic acid/water (75:10:15, by vol.) and the second in α -dipicolin/25 % ammonium hydrate/water (70:2:28, by vol.). The resulting fingerprints were compared with those from known peptidoglycan structures. Strain BH2^T possessed A1 γ , meso-diaminopimelic (*m*-Dpm)-type murein.

Total DNA was extracted by using the method of Saito & Miura (1963). The 16S rRNA genes were amplified by PCR with the following forward and reverse primers: 5'-AGAG-TTTGATCCTGGCTCAG-3' (positions 8–27 according to *Escherichia coli* numbering) and 5'-GGCTACCTTGTTAC-GACTT-3' (positions 1510–1492). The amplified DNA was cloned using a TA Cloning kit (Invitrogen) and sequenced using an ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with the following primers: 5'-GGAAACAGCTATGACCATG-3' (vector side), 5'-GAC-TACCAGGGTATCTAATC-3' (positions 805–786), 5'-AGGGTTGCGCTCGTTG-3' (positions 1115–1100) and 5'-GTAAAACGACGGCCAGT-3' (vector side), using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). There was little (2 or 3 bases) heterogeneity among the sequences (1492 bp) of several clones. The sequence of strain BH2^T was shown to be most closely related to three species of the genus *Alkalibacillus* (*Alkalibacillus haloalkaliphilus*, *Alkalibacillus filiformis* and *Alkalibacillus salilacus*; 94.1–93.6 % sequence similarity), *Thalassobacillus devorans* (94.0 %), *Filobacillus milosensis* (94.0 %) and *Tenuibacillus multivorans* (92.9 %) by FASTA analysis. The sequences of related strains retrieved from the DNA Database of Japan (DDBJ) (Miyazaki *et al.*, 2003; Pearson & Lipman, 1988; Lipman & Pearson, 1985) were aligned using the CLUSTAL W multiple sequence alignment program (Thompson *et al.*, 1994). A phylogenetic tree was reconstructed by using the neighbour-joining method (Saitou & Nei, 1987) and evaluated by bootstrap sampling (Felsenstein, 1985). Fig. 1 shows that strain BH2^T is a member of the family *Bacillaceae* and is most closely related to the three species of the genus *Alkalibacillus*, *F. milosensis* and *Tenuibacillus multivorans*. The G + C content of the total DNA of strain BH2^T, determined using the HPLC method of Tamaoka & Komagata (1984), was 35.1 ± 0.4 mol% (± SD; *n* = 5).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships between strain BH2^T and related strains. The sequence of *Virgibacillus pantothenicus* used was basically that with GenBank accession no. D78477, 16 nucleotides of which were supplemented by the corresponding bases of the sequence with GenBank accession no. X60627. Bootstrap percentages based on 1000 replicates greater than 80 % are shown. *Bacillus subtilis* JCM 1465^T (X60646) was used as an outgroup. Bar, 0.01 changes per nucleotide position. An extended neighbour-joining tree is available as Supplementary Fig. S2 in IJSEM Online.

In terms of phenotypic characteristics, there were differences between strain BH2^T and the three most-related species of the genus *Alkalibacillus* (Fritze, 1996; Jeon *et al.*, 2005; Romano *et al.*, 2005), *F. milosensis* (Schlesner *et al.*, 2001) and *Tenuibacillus multivorans* (Ren & Zhou, 2005), as summarized in Table 1. Strain BH2^T grew at pH 5.5–10.0 with optimal growth at pH 8.5–9.0, whereas *F. milosensis* and *Tenuibacillus multivorans* were neutrophilic with no growth above pH 9.0. The *Alkalibacillus* species were alkaliphilic with no growth below pH 6.5. The murein type of strain BH2^T was *m*-Dpm, as for the related genera *Alkalibacillus* and *Tenuibacillus*. *F. milosensis* was shown to possess A4 β , L-Orn-D-Glu-type murein. The major fatty acid of strain BH2^T was ai-C_{15:0} (i-C_{16:0} was the second most abundant), whereas those of the three *Alkalibacillus* species and *Tenuibacillus multivorans* were i-C_{15:0} (ai-C_{15:0} or ai-C_{17:0}) and those of *F. milosensis* were ai-C_{15:0} (i-C_{15:0}). Other differences between strain BH2^T and *F. milosensis* occurred in Gram stain, oxidase reaction, hydrolysis of DNA and hippurate and sensitivity to streptomycin, and other differences between strain BH2^T and *Tenuibacillus multivorans* were in hydrolysis of casein, gelatin and aesculin. An alignment of 16S rRNA gene sequences of members of the genera discussed above as well as *Gracilibacillus*, *Halobacillus*, *Virgibacillus*, etc., and some halophilic strains of the genus *Bacillus* and a phylogenetic tree reconstructed from the alignment are available as Supplementary Figs S1 and S2, respectively, in IJSEM Online. In addition, a table showing signature bases of the genera *Alkalibacillus*, *Filobacillus* and *Tenuibacillus* and strain BH2^T is available as Supplementary Table S1 in IJSEM Online. According to the phylogenetic and phenotypic characteristics, strain BH2^T represents a novel species of a new genus, for which the

name *Halalkalibacillus halophilus* gen. nov., sp. nov. is proposed.

Description of *Halalkalibacillus* gen. nov.

Halalkalibacillus (Hal.al.ka'li.ba.cil'lus. Gr. n. *hals* salt; Arabic n. *al qaliy* soda ash; L. n. *bacillus* rod; N.L. masc. n. *Halalkalibacillus* briny and alkaline media loving rods).

Cells are motile with a single polar flagellum, rod-shaped, 0.3–0.5 $\times$ 2.0–3.0 μ m. Spores are spherical, located terminally, with swollen sporangia. Gram-positive. KOH test and L-alanine aminopeptidase are negative. Cell walls contain A1 γ , *m*-Dpm-type murein. No growth occurs in media without NaCl. Alkaliphilic, mesophilic, aerobic and halophilic. Catalase- and oxidase-positive. Reduction of nitrate and gas formation are not observed. The G + C content of the total DNA is 35.1 $\pm$ 0.4 mol% ($\pm$ SD; *n* = 5). The predominant isoprenoid quinone is MK-7. Major cellular fatty acids are ai-C_{15:0}, i-C_{16:0}, ai-C_{17:0} and i-C_{15:0}. The type species is *Halalkalibacillus halophilus*.

Description of *Halalkalibacillus halophilus* sp. nov.

Halalkalibacillus halophilus (ha.lo.phi'lus. Gr. n. *hals* salt; Gr. adj. *philos* loving; N.L. masc. adj. *halophilus* salt loving).

Exhibits the following characteristics in addition to those given in the genus description. Colonies formed on agar are cream-like and opaque. Growth occurs in 5.0–25 % (w/v) NaCl, with optimum growth at 10–15 % (w/v) NaCl. Growth occurs at pH 5.5–10.0, with optimum growth at pH 8.5–9.0. Temperature range for growth is 20–40 $^{\circ}$ C, with optimum

Table 1. Differential characteristics of strain BH2^T and related taxa

Strains: 1, strain BH2^T; 2, *A. haloalkaliphilus* JCM 12303^T; 3, *A. filiformis* DSM 15448^T; 4, *A. salilacus* DSM 16460^T; 5, *F. milosensis* DSM 13259^T; 6, *Tenuibacillus multivorans* NBRC 100370^T. Data from Fritze (1996), Jeon *et al.* (2005), Ren & Zhou (2005), Romano *et al.* (2005), Schlesner *et al.* (2001) and this study (strain BH2^T and *A. haloalkaliphilus* JCM 12303^T). +, Positive; –, negative; W, weak; ND, no data.

Characteristic	1	2	3	4	5	6
Cell morphology	Short rods	Long rods	Long rods	Rods	Long rods	Rods
Colonial pigmentation	Cream	Cream–white	White transparent	Cream	White transparent	Translucent
Motility	+	+	–	+	+	+
Gram stain	+	–	+	+	–	+
KOH test	–	–	+	–	–	–
Salinity range for growth (optimum) (%)	5–25 (10–15)	0–20 (5–10)	0–18 (10)	5–20 (10–12)	2–23 (8–14)	1–20 (5)
pH range for growth (optimum)	5.5–10.0 (8.5–9.0)	7.5–10.0 (9.0–9.5)	7.0–10.0 (9.0)	7.0–9.0 (8.0)	6.5–8.9 (7.3–7.8)	6.5–9.0 (7.0–8.0)
Temperature range for growth (optimum) (°C)	20–40 (30–37)	20–40 (30–37)	15–45 (30)	15–40 (30)	–42 (33–38)	21–42 (36–41)
Acid production from D-fructose	–	ND	–	W	–	–
Catalase	+	+	W	+	+	+
Oxidase	+	+	–	–	–	+
Hydrolysis of:						
Starch	–	W	–	–	–	–
Casein	–	+	–	–	–	+
Gelatin	–	+	+	–	–	+
DNA	–	ND	–	ND	+	ND
Hippurate	–	+	–	+	+	ND
Aesculin	–	ND	ND	+	–	+
Reduction of nitrate	–	–	–	+	–	–
Sensitive to:						
Streptomycin	–	–	–	ND	+	ND
Tetracycline	–	+	–	ND	+	ND
DNA G + C content (mol%)	35.1 ± 0.4 (n = 5)	38.0	39.5	41.0	35	36.5–37
Murein type	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	L-Orn	<i>m</i> -Dpm
Isoprenoid quinone	MK-7	MK-7	MK-7, DemethylMK-6	MK-7	ND	ND
Major fatty acids	ai-C _{15:0} , i-C _{16:0}	i-C _{15:0} , ai-C _{15:0}	i-C _{15:0} , ai-C _{17:0}	i-C _{15:0} , ai-C _{15:0}	ai-C _{15:0} , i-C _{15:0}	i-C _{15:0} , ai-C _{15:0}

growth at 30–37 °C. Anaerobic growth is not observed. Acid is not produced from D-galactose, maltose, sucrose, D-trehalose, D-mannitol, D-fructose, D-glucose or D-xylose. Starch, gelatin, casein, DNA, hippurate, aesculin, pullulan and Tween 80 are not hydrolysed. Sensitive to ampicillin, bacitracin, novobiocin and chloramphenicol; resistant to tetracycline, streptomycin, kanamycin and anisomycin.

The type strain is strain BH2^T (=JCM 14192^T=DSM 18494^T), which was isolated from non-saline surface soil from a garden in Niiza City, Saitama Prefecture, Japan.

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References

- Dussault, H. P. (1955). An improved technique for staining red-halophilic bacteria. *J Bacteriol* **70**, 484–485.
- Echigo, A., Hino, M., Fukushima, T., Mizuki, T., Kamekura, M. & Usami, R. (2005). Endospores of halophilic bacteria of the family *Bacillaceae* isolated from non-saline Japanese soil may be transported by Kosa event (Asian dust storm). *Saline Systems* **1**, 8.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fritze, D. (1996). *Bacillus haloalkaliphilus* sp. nov. *Int J Syst Bacteriol* **46**, 98–101.

- Gregersen, T. (1978).** Rapid method for distinction of gram-negative from gram-positive bacteria. *Eur J Appl Microbiol Biotechnol* **5**, 123–127.
- Jeon, C. O., Lim, J. M., Lee, J. M., Xu, L. H., Jiang, C. L. & Kim, C. J. (2005).** Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **55**, 1891–1896.
- Kodaka, H., Armfield, A. Y., Lombard, G. L. & Dowell, V. R., Jr (1982).** Practical procedure for demonstrating bacterial flagella. *J Clin Microbiol* **16**, 948–952.
- Komagata, K. & Suzuki, K. (1987).** Lipid and cell wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Kushner, D. J. & Kamekura, M. (1988).** Physiology of halophilic eubacteria. In *Halophilic Bacteria*, vol. I, pp. 109–140. Boca Raton, FL: CRC Press.
- Lenore, S. C., Arnold, E. G. & Andrew, D. E. (1998).** *Standard Methods for the Examination of Water and Wastewater*, 20th edn. Washington, DC: American Public Health Association, the American Water Works Association and the Water Environment Federation.
- Lipman, D. J. & Pearson, W. R. (1985).** Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441.
- Miyazaki, S., Sugawara, H., Gojobori, T. & Tateno, Y. (2003).** DNA Data Bank of Japan (DDBJ) in XML. *Nucleic Acids Res* **31**, 13–16.
- Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C. & Tenover, R. H. (1999).** *Manual of Clinical Microbiology*, 7th edn. Washington DC: American Society for Microbiology.
- Oren, A. (2002).** *Halophilic Microorganisms and their Environments*. Dordrecht: Kluwer Academic.
- Oren, A., Ventosa, A. & Grant, W. D. (1997).** Proposed minimal standards for description of new taxa in the order Halobacteriales. *Int J Syst Bacteriol* **47**, 233–238.
- Pearson, W. R. & Lipman, D. J. (1988).** Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- Ren, P. G. & Zhou, P. J. (2005).** *Tenuibacillus multivorans* gen. nov., sp. nov., a moderately halophilic bacterium isolated from saline soil in Xin-Jiang, China. *Int J Syst Evol Microbiol* **55**, 95–99.
- Romano, I., Lama, L., Nicolaus, B., Gambacorta, A. & Giordano, A. (2005).** *Alkalibacillus filiformis* sp. nov., isolated from a mineral pool in Campania, Italy. *Int J Syst Evol Microbiol* **55**, 2395–2399.
- Saito, H. & Miura, K. (1963).** Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.
- Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schleifer, K. H. (1985).** Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.
- Schleifer, K. H. & Kandler, O. (1972).** Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Schlesner, H., Lawson, P. A., Collins, M. D., Weiss, N., Wehmeyer, U., Völker, H. & Thomm, M. (2001).** *Filobacillus milensis* gen. nov., sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glu-type peptidoglycan. *Int J Syst Evol Microbiol* **51**, 425–431.
- Smibert, R. M. & Krieg, N. R. (1981).** General characterization. In *Manual of Methods for General Microbiology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Smibert, R. M. & Krieg, N. R. (1994).** Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt. Washington, DC: American Society for Microbiology.
- Tamaoka, J. (1986).** Analysis of bacterial menaquinone mixtures by reverse-phase high-performance liquid chromatography. *Methods Enzymol* **123**, 251–256.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–134.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.