Alicyclobacillus vulcanalis sp. nov., a thermophilic, acidophilic bacterium isolated from Coso Hot Springs, California, USA

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A thermo-acidophilic Gram-positive bacterium, strain CsHg2^T, which grows aerobically at 35–65 °C (optimum 55 °C) and at pH 2.0–6.0 (optimum 4.0), was isolated from a geothermal pool located in Coso Hot Springs in the Mojave Desert, California, USA. Phylogenetic analysis of 16S rRNA gene sequences showed that this bacterium was most closely related to the type strains of *Alicyclobacillus acidocaldarius* (97.8 % identity) and *Alicyclobacillus sendaiensis* (96.9 %), three Japanese strains denoted as UZ-1, KHA-31 and MIH 332 (96.1–96.5 %) and *Alicyclobacillus* genomic species FR-6 (96.3 %). Phenotypic characteristics including temperature and pH optima, G + C composition, acid production from a variety of carbon sources and sensitivity to different metal salts distinguished CsHg2^T from *A. acidocaldarius*, *A. sendaiensis* and FR-6. The cell lipid membrane was composed mainly of ω -cyclohexyl fatty acid, consistent with membranes from other *Alicyclobacillus* species. Very low DNA–DNA hybridization values between CsHg2^T and the type strains of *Alicyclobacillus* indicate that CsHg2^T represents a distinct species. On the basis of these results, the name *Alicyclobacillus vulcanalis* sp. nov. is proposed for this organism. The type strain is CsHg2^T (ATCC BAA-915^T = DSM 16176^T).

Members of the genus *Alicyclobacillus* are heterotrophic, Gram-positive, low-G + C organisms that inhabit mostly acidic geothermal environments. They were formerly assigned to the genus *Bacillus* but were reclassified as a separate genus because of their distinct 16S rRNA gene sequences and unique ω -alicyclic fatty acid lipid membrane component (Wisotzkey *et al.*, 1992). Five of the eight described species of *Alicyclobacillus*, namely *Alicyclobacillus acidocaldarius*, *Alicyclobacillus acidoterrestris* (Wisotzkey *et al.*, 1992), *Alicyclobacillus hesperidum* (Albuquerque *et al.*, 2000), *Alicyclobacillus acidiphilus* (Matsubara *et al.*, 2002) and *Alicyclobacillus sendaiensis* (Tsuruoka *et al.*, 2003) contain ω -cyclohexane fatty acids, whereas *Alicyclobacillus cycloheptanicus* (Deinhard *et al.*, 1987) and *Alicyclobacillus herbarius* (Goto *et al.*, 2002) contain ω -cycloheptane fatty acids. A novel species, *Alicyclobacillus pomorum*, was described that did not possess ω -alicyclic fatty acids (Goto *et al.*, 2003). The description of the cellular fatty acid profile of the genus has since been amended to include organisms that possess straight- and branched-chain fatty acids instead of

ω -alicyclic fatty acids (Goto *et al.*, 2003). Three Japanese strains, designated UZ-1, MIH 332 and KHA-31, were also found to contain ω -cyclohexane fatty acids and, by virtue of 16S rRNA gene sequence analysis and DNA–DNA hybridization assays, were denoted as a genomic species of *Alicyclobacillus* (Hiraishi *et al.*, 1997).

A. hesperidum and another genomic species, FR-6, were described by Albuquerque *et al.* (2000). Despite low levels of genomic DNA–DNA hybridization between strain FR-6 and *A. acidocaldarius*, this strain was designated a genomic species because there were insufficient distinguishing characteristics available to determine whether it constituted a novel species. The latest species that have been described are *A. herbarius*, which was isolated from herbal tea made from the dried flowers of hibiscus (Goto *et al.*, 2002), *A. sendaiensis*, isolated from Japanese soil, which has potential biotechnological applications as a source of heat-stable collagenase (Tsuruoka *et al.*, 2003), *A. acidiphilus*, which can cause ‘off flavour’ in orange juice (Matsubara *et al.*, 2002), and *A. pomorum*, isolated from spoiled fruit juice (Goto *et al.*, 2003).

The natural habitats for most of these organisms are notable for their unique geochemistries, often rich in metals and metal sulfide minerals. To aid in the characterization of

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these organisms, metal susceptibility tests were conducted and were found to be useful as a means of distinguishing between *Alicyclobacillus* species. Metal-based phenotypic analysis provides a new tool for comparing organisms from soil environments.

Strain CsHg2^T was isolated from pool water (78 °C, pH 1.7) obtained from an acidic geothermal pool located in Coso Hot Springs in the Mojave Desert, California, USA. The water sample was adjusted to pH 5.5 using calcium carbonate, and cells were recovered by filtration using a 0.22 µm sterile membrane. The filter was immersed in neutralized pool water and transported at ambient temperature to the laboratory. The sample was plated on solid medium using Gelrite (gellan gum; Kelco) plates. The medium employed the basal salts medium of Allen (1959) as modified by Brock *et al.* (1972) containing 0.2% (w/v) tryptone and adjusted to a final pH of 2.5 with sulfuric acid. Plates were incubated at 70 °C until growth was observed. CsHg2^T was purified by streaking for single colonies on plates three times. Pure cultures of CsHg2^T were stored according to the method described by Rolfmeier & Blum (1995). Other organisms used in the following tests were *Alicyclobacillus cycloheptanicus* ATCC 49028^T, *A. acidoterrestris* ATCC 49025^T and *A. acidocaldarius* ATCC 27009^T, which were obtained from the ATCC. *Alicyclobacillus* sp. FR-6 (= DSM 11984) and *A. hesperidum* DSM 12489^T were obtained from the DSMZ and three *Alicyclobacillus* strains, UZ-1, MIH 332 and KHA-31, were kindly provided by Dr Y. Tanimoto of Okayama University, Japan.

Morphological examination of the cells was via a phase-contrast microscope. Presence of spores was checked by spore staining and culture smears were Gram stained (Hucker & Conn, 1923). Growth of CsHg2^T at different temperatures was determined in modified Allen medium with shaking. Optical density of the culture was monitored over a 24 h period at a wavelength of 600 nm. The optimum pH for growth was determined in ATCC 573 medium [1.3 g (NH₄)₂SO₄, 0.37 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, 0.07 g CaCl₂·2H₂O, 0.02 g FeCl₃·6H₂O, 0.35% (w/v) tryptone and 0.4% (w/v) glucose per litre of solution] adjusted to different pH values using sulfuric acid. Cultures were grown at 55 °C with shaking and changes in turbidity of the cultures were monitored over a 24 h period. Acid production from selected carbohydrates was determined using API 50 CH test strips (bioMérieux) in BAM basal salts medium (Albuquerque *et al.*, 2000). Sensitivity to metal salts was determined as the occurrence of growth using initial inocula of 10⁷ cells ml⁻¹ in 10 ml modified Allen medium containing metal salts. Tubes were incubated with shaking for 24 h at 55 °C and a positive test result reflected final turbidities that exceeded initial values.

Cell biomass for lipid analysis was obtained from overnight cultures grown in ATCC 573 medium at 55 °C. Methylated ester-linked fatty acids were prepared as described by Bligh & Dyer (1959), Kates (1986) and White *et al.* (1979), and separated on a Hewlett Packard 5890 Series II GC containing

an Ultra2 HP column (50 m, 0.2 mm i.d., 0.33 µm film thickness) in split mode (44:1) with a 0.75 min purge time. Oven temperature was increased from 50 °C to 160 °C at 40 °C min⁻¹ and held for 2 min, then increased at 3 °C min⁻¹ to 300 °C and held for 30 min. Fatty acids were identified using equivalent chain-length standards (Bacterial Acid Methyl Esters CP mix; Supelco) and confirmed by GC-MS. The identity of the cyclo fatty acids was confirmed by GC-MS of their methyl esters (Oshima & Ariga, 1975) and their dimethyloxazoline derivatives (Yu *et al.*, 1989).

Genomic DNA was prepared as described by Partridge *et al.* (1993). 16S rRNA genes were PCR-amplified (Haseltine *et al.*, 2001) using primers Bac11, 5'-AGAGTTTGATCC-TGGCTCAG-3', and Bact1492, 5'-GGTTACCTTGTTA-CGACTT-3' (De Long, 1992), and purified and sequenced as described by Haseltine *et al.* (1999). Sequence identities were calculated using BESTFIT included in the Wisconsin sequence analysis package, version 8 (GCG). An alignment of 1412 nucleotides was generated using the CLUSTAL W (Thompson *et al.*, 1994) function included in BIOEDIT (Hall, 1999). A phylogenetic distance tree with bootstrap analysis from 1000 replications was generated using the neighbour-joining method included in MEGA, version 2.1 (Kumar *et al.*, 2001).

Genomic hybridization was performed as described by Urbance *et al.* (2001) with the following modifications: the hybridization buffer contained 50% formamide, and hybridization was conducted for 48 h at 45 °C on a shaking water bath, while probe binding was measured by liquid scintillation counting (Liquid Scintillation System LS 1701; Beckman). Hybridization of CsHg2^T-labelled genomic DNA was tested against all *Alicyclobacillus* species and strains mentioned above. The DNA G+C content was determined by a fluorometric method that measures thermal denaturation of DNA (Gonzalez & Saiz-Jimenez, 2002). G+C content of CsHg2^T was estimated from a calibration curve prepared by plotting the *T*_m of three known bacteria, *Bacillus subtilis* ATCC 6051^T, *A. acidocaldarius* ATCC 27009^T and *Pseudomonas auruginosa* ATCC 10145^T, against their G+C contents.

Cells of strain CsHg2^T were Gram-positive rods, 1.5–2.5 µm long and 0.4–0.7 µm wide, possessing terminal spores. CsHg2^T grew aerobically at 35–65 °C with an optimum of 55 °C and over a range of pH values from 2.0 to 6.0 with an optimum of 4.0. Phenotypic characteristics of CsHg2^T and related species of *Alicyclobacillus* (those sharing more than 94% sequence similarity) are shown in Table 1. CsHg2^T hydrolysed starch on starch plates, was oxidase and catalase negative and grew on solid media with up to 2% (w/v) sodium chloride. Bacterial colonies were semi-transparent to white, convex and about 1 mm in diameter after growth for 28 h at 55 °C. CsHg2^T produced acid from a variety of pentoses and hexoses, some disaccharides and sugar alcohols (Table 1). Some results for acid production from various carbohydrates did not match the results given by Albuquerque *et al.* (2000) and Tsuruoka

Table 1. Phenotypic characteristics of CsHg₂^T and other *Alicyclobacillus* species

Strains: 1, CsHg₂^T; 2, *A. acidocaldarius* ATCC 27009^T; 3, *Alicyclobacillus* sp. FR-6 (=DSM 11984); 4, *A. sendaiensis* ATCC BAA-609^T; 5, *A. hesperidum* DSM 12489^T; 6, *A. acidoterrestris* ATCC 49025^T. +, Positive result; –, negative result; W, weakly positive; NR, not reported. All strains were positive for the oxidase test and acid production from glycerol, L-arabinose, galactose, D-glucose, mannose and cellobiose. All strains were negative for acid production from D-arabinose, L-xylose, adonitol, sorbose, dulcitol, methyl α -D-mannoside, N-acetylglucosamine, amygdalin, aesculin, inulin, melezitose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate and 2-ketogluconate. For acid production, data for columns 1, 2, 5 and 6 are from this study; column 3 from Albuquerque *et al.* (2000) and column 4 from Tsuruoka *et al.* (2003).

Characteristic	1	2	3	4	5	6
Gram reaction	+	+	+	–	+	+
Temperature range (°C)	35–65	45–70	>40, <70	40–65	>35, <60	35–55
Optimum temperature (°C)	55	65	60–63	55	50–53	42–53
pH range	2.0–6.0	2.0–6.0	>2.0, <6.0	2.5–6.5	>2.0, <6.0	2.2–5.8
Optimum pH	4.0	3.0–4.0	3.5–4.0	5.5	3.5–4.0	4.0
G + C content (mol%)	62.0	60.3	60.3	62.3	53.3	51.6–53.3
Catalase reaction	–	+	W	–	W	+
Growth in 5% NaCl	–	–	NR	–	–	+
Hydrolysis of starch	+	+	–	NR	+	–
Acid production from:						
Erythritol	–	–	–	–	–	+
Ribose	+	+	–	+	–	+
D-Xylose	+	+	–	+	+	+
Methyl β -xyloside	–	–	–	–	+	–
D-Fructose	+	+	+	+	–	+
Rhamnose	–	W	–	–	–	+
Inositol	+	–	–	–	–	+
Mannitol	+	–	+	+	+	+
Sorbitol	–	–	–	–	–	+
Methyl β -D-glucoside	W	–	–	+	–	–
Arbutin	W	–	–	+	–	–
Salicin	–	–	–	+	W	–
Lactose	–	+	+	+	+	–
Melibiose	+	+	–	–	–	–
Sucrose	+	+	+	+	+	–
Trehalose	+	+	+	+	+	–
D-Raffinose	+	+	+	+	–	–
Glycogen	+	+	–	+	+	–
Xylitol	–	–	–	–	–	+
β -Gentiobiose	–	–	–	–	+	+
D-Turanose	+	+	+	+	+	–
5-Ketogluconate	W	W	–	W	–	–

et al. (2003), the reasons for which were discussed by Goto *et al.* (2002). The total fatty acids of CsHg₂^T included 46 % ω -cyclohexyl 17:0 and 22.6 % ω -cyclohexyl 19:0 (Table 2), placing CsHg₂^T in the group of *Alicyclobacillus* that possess ω -cyclohexyl fatty acids.

Metal susceptibility tests were conducted to aid in the characterization of CsHg₂^T. *A. acidocaldarius* ATCC 27009^T and strain FR-6 cluster with CsHg₂^T on the basis of 16S rRNA gene phylogenetic analysis. These three strains were all isolated from acidic, thermal solfataric environments that have unique geochemistries often characterized by high concentrations of different metal

salts. CsHg₂^T was isolated from a hot spring that contained significant amounts of iron, other trace metals and sulfur. Consequently, a metals sensitivity assay was used to provide a phenotypic survey of characteristics likely to be involved in tolerance to environmental levels of metals and metal salts in natural habitats. Based on this assay, CsHg₂^T can be distinguished from strain FR-6 by its response to eight of the nine metal salts tested, and from *A. acidocaldarius* ATCC 27009^T based on five of the nine metal salts tested (Table 3).

The 16S rRNA gene sequence of CsHg₂^T shared 90.6–97.8 % similarity (over 1458 nt) with all *Alicyclobacillus*

Table 2. Cellular fatty acid composition (%) of CsHg2^T and different *Alicyclobacillus* species

Strains: 1, CsHg2; 2, *A. acidocaldarius* ATCC27009^T; 3, *Alicyclobacillus* sp. FR-6; 4, *A. acidoterrestris* DSM 3923^T; 5, *A. hesperidum* DSM 12489^T; 6, *A. sendaiensis* ATCC BAA-609^T. Tr, Trace; —, not detected. Data for columns 2 and 4 are from Matsubara *et al.* (2002), columns 3 and 5 from Albuquerque *et al.* (2000) and column 6 from Tsuruoka *et al.* (2003).

Fatty acid	1	2	3	4	5	6
15:0 iso	1.5	1.4	1.7	Tr	5.4	1.1
15:0 anteiso	0.7	—	0.8	Tr	6.6	1.5
16:0 iso	4.2	1.4	1.4	—	0.9	1.6
16:0	7.8	—	0.9	2.5	2.1	4.9
17:0 iso	8.3	1.9	8.3	1.2	4.9	4.5
17:0 anteiso	8.5	2.3	8.4	4.1	10.3	10.5
ω -Cyclohexane 17:0	46.2	78.0	51.8	65.8	56.8	44.1
ω -Cyclohexane 19:0	22.8	16.0	26.7	24.0	13.3	30.2

Table 3. Sensitivity of CsHg2^T, *Alicyclobacillus acidocaldarius* ATCC 27009^T and *Alicyclobacillus* sp. FR-6 to different metal salts

Strains: 1, CsHg2^T; 2, *Alicyclobacillus* sp. FR-6; 3, *A. acidocaldarius* ATCC 27009^T. S, Sensitive; R, resistant.

Metal compound (concentration)	1	2	3
Molybdc acid (0.2 mM)	R	S	S
Zinc chloride (40 mM)	R	S	R
Mercuric chloride (0.002 mM)	R	S	S
Cupric chloride (8 mM)	S	R	S
Cobalt chloride (1 mM)	S	R	S
Sodium tungstate (0.01 mM)	S	S	R
Sodium iodide (10 mM)	S	R	S
Sodium selenate (10 mM)	R	S	S
Cadmium chloride (0.8 mM)	S	R	R

species and strains. It shared the closest identity with *A. acidocaldarius* ATCC 27009^T (97.8 %), *A. sendaiensis* JCM11817 (96.9 %), three previously described but unresolved strains from Japanese hot springs (96.15–96.5 %) and the genomic species FR-6 (96.33 %). It shared 94.3 % identity with *A. hesperidum* DSM 12489^T and 94 % with

A. acidoterrestris ATCC 49025^T. Phylogenetic analysis revealed the relationship between CsHg2^T and the different *Alicyclobacillus* species (Fig. 1). To clarify the extent of relatedness between CsHg2^T and these species, genomic DNA–DNA hybridization was conducted. Although the 16S rRNA gene sequence of CsHg2^T shared the highest relatedness to *A. acidocaldarius* ATCC 27009^T, DNA–DNA relatedness between these two strains was only 9 %. DNA–DNA hybridization values of CsHg2^T did not exceed 24 % for any of the *Alicyclobacillus* species tested. Strain FR-6 exhibited the highest binding to CsHg2^T, at 24 %. These results indicate that CsHg2^T is distinct from other alicyclobacilli and can be classified as a separate species (Wayne *et al.*, 1987). The G + C content of CsHg2^T was 62 mol%. This falls within the range of values of the different *Alicyclobacillus* species (51.6–62.3 mol%).

Description of *Alicyclobacillus vulcanalis* sp. nov.

Alicyclobacillus vulcanalis (vul.ca.na'lis. L. masc. adj. *vulcanalis* of Vulcan, belonging to Vulcan, Roman god of fire and metal working).

Aerobic, Gram-positive, spore-forming rods measuring 1.5–2.5 × 0.4–0.7 µm. Oxidase and catalase negative, grows in 2 % NaCl and utilizes starch. Colonies appear semi-transparent to white and grow to 1 mm diameter in 28 h at 55 °C in modified Allen medium. ω -Cyclohexyl fatty acid is the major fatty acid component of the cell. Grows at a temperature range of 35–65 °C (optimum 55 °C) and over a pH range of 2.0–6.0 (optimum 4.0). Resistant to 0.2 mM molybdc acid 0.002 mM mercuric chloride, 10 mM sodium selenate and 40 mM zinc chloride and sensitive to 8 mM cupric chloride, 1 mM cobalt chloride, 10 mM sodium iodide and 0.8 mM cadmium chloride. Produces acid from glycerol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, inositol, mannitol, cellobiose, maltose, melibiose, sucrose, trehalose, D-raffinose, glycogen and D-turanose, but not from D-arabinose, L-xylose, adonitol, methyl β -xyloside, L-sorbose, rhamnose, sorbitol, dulcitol, methyl α -D-mannoside, N-acetylglucosamine, amygdalin, aesculin, salicin, lactose, inulin, melezitose, starch, xylitol, β -gentiobiose, D-lyxose, D-tagalose, D-fucose, L-fucose, D-arabitol, L-arabitol,

Fig. 1. Phylogenetic distance tree derived from comparison of the 16S rRNA gene sequences of different *Alicyclobacillus* species and related taxa. The tree was prepared using the neighbour-joining method from an alignment of 1412 bases. Bootstrap analysis was conducted for 1000 repetitions and percentages are indicated at the nodes. *Geobacillus stearothermophilus* is used as the outgroup. Bar, 0.02 substitutions per site.

gluconate and 2-ketogluconate. The DNA G + C content is 62 mol%.

The type strain, CsHg2^T (=ATCC BAA-915^T=DSM 16176^T), was isolated from a hot spring in Coso Hot Springs in the Mojave Desert, California, USA.

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References

- Albuquerque, L., Rainey, F. A., Chung, A. P., Sunna, A., Nobre, M. F., Grote, R., Antranikian, G. & da Costa, M. S. (2000). *Alicyclobacillus hesperidum* sp. nov. and a related genomic species from solfataric soils of São Miguel in the Azores. *Int J Syst Evol Microbiol* **50**, 451–457.
- Allen, M. B. (1959). Studies with *Cyanidium caldarium*, an anomalously pigmented chlorophyte. *Arch Mikrobiol* **32**, 270–277.
- Bligh, E. G. & Dyer, W. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
- Brock, T., Brock, K., Belly, R. & Weiss, R. (1972). *Sulfolobus*: a new genus of sulfur oxidizing bacteria living at low pH and high temperature. *Arch Mikrobiol* **84**, 54–68.
- De Long, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci U S A* **89**, 5685–5689.
- Deinhard, G., Saar, J., Krischke, W. & Poralla, K. (1987). *Bacillus cycloheptanicus* sp. nov., a new thermophile containing ω -cycloheptane fatty acids. *Syst Appl Microbiol* **10**, 47–53.
- Gonzalez, J. M. & Saiz-Jimenez, C. (2002). A fluorometric method for the estimation of G + C mol% content in microorganisms by thermal denaturation temperature. *Environ Microbiol* **4**, 770–773.
- Goto, K., Matsubara, H., Mochida, K., Matsumura, T., Hara, Y., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus herbarius* sp. nov., a novel bacterium containing ω -cycloheptane fatty acids, isolated from herbal tea. *Int J Syst Evol Microbiol* **52**, 109–113.
- Goto, K., Mochida, K., Asahara, M., Suzuki, M., Kasai, H. & Yokota, A. (2003). *Alicyclobacillus pomorum* sp. nov., a novel thermo-acidophilic, endospore-forming bacterium that does not possess ω -alicyclic fatty acids, and emended description of the genus *Alicyclobacillus*. *Int J Syst Evol Microbiol* **53**, 1537–1544.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Haseltine, C., Montalvo-Rodriguez, R., Carl, A., Bini, E. & Blum, P. (1999). Extragenic pleiotropic mutations that repress glycosyl hydrolase expression in the hyperthermophilic archaeon *Sulfolobus solfataricus*. *Genetics* **152**, 1353–1361.
- Haseltine, C., Hill, T., Montalvo-Rodriguez, R., Kemper, S., Shand, R. & Blum, P. (2001). Secreted euryarchaeal microhalocins kill hyperthermophilic crenarchaea. *J Bacteriol* **183**, 287–291.
- Hiraishi, A., Inagaki, K., Tanimoto, Y., Iwasaki, M., Kishimoto, N. & Tanaka, H. (1997). Phylogenetic characterization of a new thermoacidophilic bacterium isolated from hot springs in Japan. *J Gen Appl Microbiol* **43**, 295–304.
- Hucker, G. & Conn, H. (1923). *Methods of Gram Staining*. New York State Agricultural Experiment Station Technical Bulletin no. 93.
- Kates, M. (1986). Techniques of lipidology: isolation, analysis and identification of lipids. In *Laboratory Techniques in Biochemistry and Molecular Biology*, vol. 3, part 2. Edited by R. H. Burdon & P. H. van Kippenberg. New York: Elsevier.
- Kumar, S., Tamura, K., Jakobsen, I. & Nei, M. (2001). MEGA2: Molecular Evolutionary Genetics Analysis software. Tempe, AZ: Arizona State University.
- Matsubara, H., Goto, K., Matsumura, T., Mochida, K., Iwaki, M., Niwa, M. & Yamasato, K. (2002). *Alicyclobacillus acidiphilus* sp. nov., a novel thermo-acidophilic, ω -alicyclic fatty acid-containing bacterium isolated from acidic beverages. *Int J Syst Evol Microbiol* **52**, 1681–1685.
- Oshima, M. & Ariga, T. (1975). Omega-cyclohexyl fatty acids in acidophilic thermophilic bacteria. Studies on their presence, structure, and biosynthesis using precursors labeled with stable isotopes and radioisotopes. *J Biol Chem* **250**, 6963–6968.
- Partridge, J., King, J., Krska, J., Rockabrand, D. & Blum, P. (1993). Cloning, heterologous expression, and characterization of the *Erysipelothrix rhusiopathiae* DnaK protein. *Infect Immun* **61**, 411–417.
- Rolfsmeier, M. & Blum, P. (1995). Purification and characterization of a maltase from the extremely thermophilic crenarchaeote *Sulfolobus solfataricus*. *J Bacteriol* **177**, 482–485.
- 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.
- Tsuruoka, N., Isono, Y., Shida, O., Hemmi, H., Nakayama, T. & Nishino, T. (2003). *Alicyclobacillus sendaiensis* sp. nov., a novel acidophilic, slightly thermophilic species isolated from soil in Sendai, Japan. *Int J Syst Evol Microbiol* **53**, 1081–1084.
- Urbance, J. W., Bratina, B. J., Stoddard, S. F. & Schmidt, T. M. (2001). Taxonomic characterization of *Ketogulonigenium vulgare* gen. nov., sp. nov. and *Ketogulonigenium robustum* sp. nov., which oxidize L-sorbose to 2-keto-L-gulonic acid. *Int J Syst Evol Microbiol* **51**, 1059–1070.
- Wayne, L., Brenner, D., Colwell, R. & 9 other authors (1987). Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
- White, D., Davis, W., Nickels, J., King, J. & Bobbie, R. (1979). Determination of the sedimentary microbial biomass by extractable liquid phosphate. *Oecologia* **40**, 51–62.
- Wisotzkey, J. D., Jurtshuk, P., Jr, Fox, G. E., Deinhard, G. & Poralla, K. (1992). Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *Int J Syst Bacteriol* **42**, 263–269.
- Yu, Q., Lui, B., Zhang, J. & Huang, Z. (1989). Location of double bonds in fatty acids of fish oil and rat testis lipids. Gas chromatography-mass spectrometry of the oxazoline derivatives. *Lipids* **24**, 79–83.