Halolactibacillus halophilus gen. nov., sp. nov. and *Halolactibacillus miurensis* sp. nov., halophilic and alkaliphilic marine lactic acid bacteria constituting a phylogenetic lineage in *Bacillus* rRNA group 1

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Eleven novel strains of marine-inhabiting lactic acid bacteria that were isolated from living and decaying marine organisms collected from a temperate area of Japan are described. The isolates were motile with peritrichous flagella and non-sporulating. They lacked catalase, quinones and cytochromes. Fermentation products from glucose were lactate, formate, acetate and ethanol. Lactate yield as percentage conversion from glucose was affected by the pH of the fermentation medium: ~55 % at the optimal growth pH of 8.0, greater than ~70 % at pH 7.0 and less than ~30 % at pH 9.0. The molar ratio of the other three products was the same at each cultivation pH, approximately 2 : 1 : 1. Carbohydrates and related compounds were aerobically metabolized to acetate and pyruvate as well as lactate. The isolates were slightly halophilic, highly halotolerant and alkaliphilic. The optimum NaCl concentration for growth was 2.0–3.0 % (w/v), with a range of 0–25.5 %. The optimum pH for growth was 8.0–9.5, with a range of 6.0–10.0. The G + C content of the DNA was 38.5–40.7 mol%. The isolates constituted two genomic species (DNA–DNA relatedness of less than 41 %) each characterized by sugar fermentation profiles. The cell-wall peptidoglycan of both phenotypes contained *meso*-diaminopimelic acid. The major cellular fatty acids were C_{16:0} and a-C_{13:0}. Comparative sequence analysis of the 16S rRNA genes revealed that these isolates represent novel species constituting a phylogenetic unit outside the radiation of typical lactic acid bacteria and an independent line of descent within the group composed of the halophilic/halotolerant/alkaliphilic and/or alkalitolerant species in *Bacillus* rRNA group 1, with 94.8–95.1 % similarity to the genus *Paraliobacillus*, 93.7–94.1 % to the genus *Gracilibacillus* and 93.8–94.2 % to *Virgibacillus marismortui*. On the basis of possession of physiological and biochemical characteristics common to typical lactic acid bacteria within *Bacillus* rRNA group 1, chemotaxonomic characteristics and phylogenetic independence, a new genus and two species, *Halolactibacillus halophilus* gen. nov., sp. nov. and *Halolactibacillus miurensis* sp. nov., are proposed. The type strains are *Halolactibacillus halophilus* M2-2^T (=DSM 17073^T=IAM 15242^T=NBRC 100868^T=NRIC 0628^T) (G + C content 40.2 mol%) and *Halolactibacillus miurensis* M23-1^T (=DSM 17074^T=IAM 15247^T=NBRC 100873^T=NRIC 0633^T) (G + C content 38.5 mol%).

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Abbreviations: HA group, halophilic/halotolerant/alkaliphilic and/or alkalitolerant group.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M2-2^T and M23-1^T are AB196783 and AB196784, respectively.

A phylogenetic tree constructed using the maximum-likelihood method showing the relationships between the new isolates and other related bacteria, and tables detailing the products from glucose under aerobic and anaerobic cultivation conditions and the cellular fatty acid compositions of *Halolactibacillus halophilus* M2-2^T and *Halolactibacillus miurensis* M23-1^T and related taxa are available as supplementary material in IJSEM Online.

We previously reported the isolation, taxonomic characterization and phylogenetic position of a lactic acid bacterium named *Marinilactibacillus psychrotolerans* (Ishikawa *et al.*, 2003b). It was isolated from marine organisms (living sponge, decaying alga and raw shellfish) and is slightly halophilic, highly halotolerant and alkaliphilic, growing preferably under the physico-chemical conditions found in sea water [total salt concentration, 3.2–3.8% (w/v); pH 8.2–8.3 (surface)]. For this marine-inhabiting organism, we proposed the term 'marine lactic acid bacterium'. In addition to the isolation and proposal of the genus *Marinilactibacillus*, a Gram-positive, spore-forming, facultatively anaerobic, halophilic, halotolerant, 'slightly' alkaliphilic and marine-inhabiting bacterium named *Paraliobacillus ryukyuensis* was isolated from a decaying marine alga (Ishikawa *et al.*, 2002, 2003a). It produces lactate under anaerobic conditions and belongs to the phylogenetic group whose members have halophilic/halotolerant/alkaliphilic and/or alkalitolerant properties (Garabito *et al.*, 1997; Heyndrickx *et al.*, 1999; Wainø *et al.*, 1999; Lu *et al.*, 2001; Zhilina *et al.*, 2001, 2002; Yoon *et al.*, 2002) within *Bacillus* rRNA group 1 (Ash *et al.*, 1991) in the phyletic assemblage of bacteria classically defined as the genus *Bacillus*. Most of the member species of this phylogenetic group were isolated from saline or hypersaline environments: sediments of saline lakes, hypersaline soils, solar salterns, mud of deep-sea ridge and salt fields.

In the course of isolating a marine-inhabiting lactic acid bacterium, novel halophilic and alkaliphilic lactic acid bacteria belonging to this halophilic/halotolerant/alkaliphilic and/or alkalitolerant group (henceforth referred to as the HA group) were isolated from decaying marine algae and a living sponge. Here we describe the isolation, taxonomic characterization and phylogenetic positions of the isolates, for which the names *Halolactibacillus halophilus* gen. nov., sp. nov. and *Halolactibacillus miurensis* sp. nov. are proposed.

The samples for the isolation of lactic acid bacteria were collected from Oura beach (35° 08' N 139° 40' E) on the Miura Peninsula in Kanagawa Prefecture, in the middle of the Japanese mainland, a temperate area, in July 1998. The samples collected were living, fresh or decaying sponges, algae, shellfish, crabs and fish.

The isolates of lactic acid bacteria were obtained by enrichment culture in a 7% NaCl GYPB (glucose-yeast extract-peptone-beef extract) isolation broth and pore-plating on a 7% NaCl GYPB isolation agar (1.3% agar). The compositions of the media and the method used for isolation have been described previously (Ishikawa *et al.*, 2003b). Briefly, the strains were isolated by successive enrichment and by plating. In the first enrichment culture, a small piece of the sample was soaked in medium immediately after collection and then an inoculated broth was incubated at 30 °C for 3 days. In the second enrichment culture, a broth medium was inoculated with a portion of the first enrichment broth culture, whose pH had decreased to below 7.0, and incubated anaerobically at 30 °C for 2 days.

Eleven bacterial strains were isolated from three kinds of decaying marine alga and a living sponge. Four isolates (M2-1, M2-2^T, M2-3 and M2-4) were obtained from a decaying alga. One isolate, M9-1, was from another decaying alga. Another isolate, M13-1, was from a living sponge. Five isolates (M23-1^T, M23-2, M23-3, M23-4 and M23-5) were from a third kind of alga.

Marinilactibacillus psychrotolerans M13-2^T (Ishikawa *et al.*, 2003b), *Paraliobacillus ryukyuensis* O15-7^T (Ishikawa *et al.*, 2002, 2003a), *Amphibacillus xylanus* NRIC 1994^T (Niimura *et al.*, 1990), *Amphibacillus fermentum* DSM 13869^T (Zhilina *et al.*, 2001), *Amphibacillus tropicus* DSM 13870^T (Zhilina *et al.*, 2001), *Gracilibacillus halotolerans* DSM 11805^T (Wainø *et al.*, 1999) and *Gracilibacillus dipsosauri* DSM 11125^T (Lawson *et al.*, 1996; Wainø *et al.*, 1999) were used as reference strains.

For cultivation and taxonomic characterization, a 2.5% NaCl GYPF broth, composed of 10 g glucose, 5 g yeast extract (Oriental Yeast), 5 g Polypeptone (Nippon Seiyaku), 5 g Extract Bonito (fish extract; Wako Pure Chemical), 1 g K₂HPO₄, 25 g NaCl, 1 g sodium thioglycolate, 5 ml salt solution [(ml⁻¹): 40 mg MgSO₄·7H₂O, 2 mg MnSO₄·4H₂O and 2 mg FeSO₄·7H₂O] (Okada *et al.*, 1992), and distilled water to 1000 ml, was used as the basal medium unless otherwise stated. The medium was adjusted to pH 8.5 and sterilized by filtration. When a larger cell mass was needed for cellular component analysis, the concentration of K₂HPO₄ in the 2.5% NaCl GYPF broth was increased from 0.1 to 1% to buffer the cultivation medium and improve growth (2.5% NaCl GYPF broth). The isolates were stored at 5 °C in this medium and transferred at 1-month intervals as described previously (Ishikawa *et al.*, 2003b). The isolates were grown by standing cultivation at 30 °C unless otherwise stated. Anaerobic cultivation was conducted by using AnaeroPack-Kenki (CO₂-generated; Mitsubishi Gas Chemical), as described previously (Ishikawa *et al.*, 2003b). For cultivation of the reference strains the following media were used: *Marinilactibacillus psychrotolerans* M13-2^T and *Paraliobacillus ryukyuensis* O15-7^T, the same medium as for the isolates; *Amphibacillus xylanus* NRIC 1994^T, 2.5% NaCl GYPF broth without NaCl, pH 9.0; *Amphibacillus fermentum* DSM 13869^T and *Amphibacillus tropicus* DSM 13870^T, 2.5% NaCl GYPF broth but without NaCl and salt solution, and addition of (l⁻¹): 0.1 g MgCl₂, 0.2 g KCl, 50.4 g NaHCO₃ and 63.6 g Na₂CO₃, pH 9.5; *G. halotolerans* DSM 11805^T and *G. dipsosauri* DSM 11125^T, marine broth 2216 (Difco) with addition of 10 g glucose l⁻¹ and 5 g KCl l⁻¹. All media were sterilized by filtration. *Marinilactibacillus psychrotolerans* M13-2^T and *Paraliobacillus ryukyuensis* O15-7^T were cultivated in the same way as the isolates. *Amphibacillus* species were grown by standing cultivation at 37 °C and *Gracilibacillus* species by shaking at 30 °C.

Cellular morphology, cultural characteristics, motility, Gram staining and flagellation were characterized as described previously (Ishikawa *et al.*, 2002). Spore formation was examined microscopically for cultures grown at 37 °C

on 2.5 % NaCl GYPF agar, marine agar 2216 with or without 5 p.p.m. Mn^{2+} (MnSO_4), and 2 % NaCl yeast extract salts agar (pH 8.5) composed of 5 g yeast extract, 20 g NaCl, 5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g CaCl_2 , 1 g K_2HPO_4 , 5 ml salt solution (see 2.5 % NaCl GYPF broth) and 15 g agar in 1 l distilled water, by using the method described previously (Ishikawa *et al.*, 2002). Aerobic utilization of glucose was examined by using 2.5 % NaCl GCY broth, composed of 10 g glucose, 5 g Vitamin assay Casamino acids (Difco), 0.5 g yeast extract, 25 g NaCl, 1 g K_2HPO_4 , 5 ml salt solution and distilled water in 1 l volume, as described previously (Ishikawa *et al.*, 2002). Aerobic cultivation was conducted by shaking in a cotton-plugged, L-shaped test tube containing 5 ml of the medium, on a reciprocal shaker (125 strokes min^{-1}).

Catalase activity was examined by observing the evolution of bubbles upon addition of 3 % H_2O_2 solution to aerobically or anaerobically grown cells viewed under a stereoscopic microscope. Oxidase activity, growth behaviour as a function of oxygen concentration and sugar fermentation were studied as described previously (Ishikawa *et al.*, 2003b). Production of acid was scored as positive when the titre of 0.1 M NaOH per 5 ml culture broth was ≥ 0.7 ml, as weakly positive when the titre was ≥ 0.5 ml and < 0.7 ml, and as negative for titres < 0.5 ml. Tests for nitrate reduction, and production of gas from glucose, ammonia from arginine and dextran from glucose were conducted as described by Okada *et al.* (1992). Hydrolysis of DNA (DNase test agar; Difco), casein and gelatin (Difco) was tested using agar plate methods, as described by Gerhardt *et al.* (1994). Media used for the investigation of these conventional taxonomic features were supplemented with 2.5 % NaCl and adjusted to pH 8.5.

The following studies were conducted as described previously (Ishikawa *et al.*, 2003b): growth optima and ranges of NaCl concentration, pH and temperature, and maximum and minimum concentrations of NaCl, growth temperature and pH. Growth optima for these physiological conditions were determined from the maximum specific growth rate, μ_{max} (h^{-1}).

Fermentation products from glucose consumed were analysed by HPLC as described previously (Ishikawa *et al.*, 2003b). The amount of glucose was estimated by using the Somogyi method (Somogyi, 1945) or enzymically with glucose oxidase (Wako Pure Chemical). The isomeric forms of lactic acid produced were determined enzymically with D- and L-lactate dehydrogenases (Roche Diagnostics).

Products from glucose were investigated during aerobic and anaerobic cultivation. Aerobic cultivation was conducted as described above. AnaeroPack-Keep (not CO_2 -generated; Mitsubishi Gas Chemical) was used for anaerobic cultivation. Products were analysed by HPLC as described previously (Ishikawa *et al.*, 2003b). To investigate the effect of the initial pH of the anaerobic cultivation medium on the composition of products from glucose, cultivation was performed in 2.5 % NaCl GYPF broth buffered with

100 mM HEPES, with the initial pH adjusted to 7.0, 8.0 or 9.0. To minimize the effect of any decrease in pH during the fermentation, the products were analysed when the OD_{660} of the culture reached about 0.20.

Analysis of fatty acids and detection of respiratory quinones were performed by using previously described methods (Ishikawa *et al.*, 2003b). The presence of diaminopimelic acid was determined by TLC by using the method of Hasegawa *et al.* (1983), as described by Okada *et al.* (1992). The presence of cytochrome was determined by spectrophotometry using the method described by Ohama (1982) and Niimura *et al.* (1987). Amounts of 300 mg (dry weight) and 3 g (wet weight) of cells were used for the detection of respiratory quinones and cytochromes, respectively. Cells were cultivated aerobically by shaking.

DNA base composition was determined by analysing deoxyribonucleosides by reversed-phase HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization was performed using the fluorometric method of Ezaki *et al.* (1989). For these experiments, total genomic DNA was prepared according to the combined methods of Marmur (1961) and Saito & Miura (1963).

Almost complete sequences of 16S rRNA genes were amplified by PCR using the primers 20F (5'-AGTTTGATCATG-GCTCA-3', positions 10–26) and 1540R (5'-AAGGAGGT-GATCCAACCGCA-3', positions 1541–1522) (*Escherichia coli* numbering system; Brosius *et al.*, 1978), as described previously (Yanagi & Yamasato, 1993; Ishikawa *et al.*, 2003b). The amplified products were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing ready reaction kit and an ABI PRISM model 310 Genetic Analyzer (Perkin Elmer). The following five primers were used: 20F, 1540R, 350F (5'-CCTACGGGAGGCAGCAGT-3', positions 341–358), 800F (5'-GTAGTCCACGCCGTAAACGA-3', positions 800–819) and 900R (5'-CGGCCGTACTCCCCAGGCGG-3', positions 898–879). Percentage similarities among the isolates, the members of the HA group and other typical lactic acid bacteria were calculated for aligned DNA sequences of 1452–1468 bases in length using the program GENETIX (SDC Software Development). Known sequences were retrieved from public databases and aligned with the newly determined sequences using the program CLUSTAL_X (Thompson *et al.*, 1997). From the resulting multiple-sequence alignment, hypervariable regions at positions 66–103 (V1 region) and 1436–1456 (V5 region) were omitted to avoid analytical errors in constructing the tree. Unrooted phylogenetic trees were reconstructed by using the maximum-likelihood method of Felsenstein (1981), as implemented in the program fastDNAmI (Olsen *et al.*, 1984), and also from the K_{nuc} values (Kimura, 1980) by using the neighbour-joining method of Saitou & Nei (1987). The stability of the groupings was estimated by bootstrap analysis (1000 replications from the neighbour-joining dataset; Felsenstein, 1985).

All 11 newly isolated strains exhibited similar taxonomic features. Cultural and morphological characteristics and

Fig. 1. Photomicrographs of cells and peritrichous flagella of *Halolactibacillus halophilus* M2-2^T (a) and *Halolactibacillus miurensis* M23-1^T (b) grown anaerobically at 30 °C for 2 days on 2.5 % NaCl GYPFK agar. Bars, 2 µm.

other taxonomic features are given in the descriptions of the new genus and species. Cells and peritrichous flagella of strains M2-2^T and M23-1^T are shown in Fig. 1. Spores were not produced when the cells were cultivated on 2.5 % NaCl GYPF agar, marine agar 2216 with or without 5 p.p.m. Mn²⁺ or 2 % NaCl yeast extract salts agar. Catalase was negative for cells that were grown aerobically or anaerobically in 2.5 % NaCl GYPF broth, on the same medium with the concentration of glucose reduced to 0.1 %, or on 2.5 % NaCl GYPF agar or 2 % NaCl yeast extract salts agar.

Under aerobic conditions, no growth was observed in 2.5 % NaCl GCY broth from which glucose was excluded. That is, in the stationary phase, the OD₆₆₀ of cultures grown in 2.5 % NaCl GCY broth was about 0.20, and the OD₆₆₀ of those grown in the absence of glucose was less than 0.02. Also, in

2.5 % NaCl GYPF broth, growth was weak when the initial glucose concentration was decreased to 0.1 %, and did not occur when glucose was omitted. Growth occurred, but with little vigour, on a 2.5 % NaCl GYPF agar plate exposed to the atmosphere. The amount of growth was not affected by the oxygen concentration in the medium: the density and size of colonies that developed on semisolid medium that was evenly inoculated and statically incubated were uniform from the surface to the bottom.

A fairly wide range of carbohydrates, sugar alcohols and related carbon compounds was fermented (Table 1). These fermentation experiments revealed the presence of two phenotypes within the 11 isolates, which we designated phenotype 1 and phenotype 2. The six isolates M2-1, M2-2^T, M2-3, M2-4, M9-1 and M13-1 had phenotype 1, and the five isolates M23-1^T, M23-2, M23-3, M23-4 and M23-5 had phenotype 2. Phenotypes 1 and 2 were distinguished on the basis of the following utilization pattern of carbon compounds: phenotype 1 strains did not ferment L-arabinose, D-xylose, D-melezitose or inulin but did ferment glycerol, whereas phenotype 2 strains fermented these sugars and weakly fermented glycerol. Sodium gluconate was fermented without the production of gas by all the isolates.

The optimum NaCl concentrations for growth were between 2.0 % (0.34 M) and 3.0 % (0.51 M) (1.5–3.0 % for strains M9-1 and M23-5) for phenotype 1 and between 2.5 % (0.43 M) and 3.0 % for phenotype 2. The maximum specific growth rates, $\mu_{\max}$ (h⁻¹), of strain M2-2^T, phenotype 1, were 0.18 in 0 %, 0.22 in 0.5 %, 0.30 in 1.0 %, 0.46 in 1.5 %, 0.48 in 2.0 %, 0.54 in 2.5 %, 0.40 in 3.0 %, 0.40 in 3.75 %, and 0.40 in 5.0 % NaCl. Those of strain M23-1^T, phenotype 2, were 0.40 in 0 %, 0.44 in 0.5 %, 0.56 in 1.0 %, 0.56 in 1.5 %, 0.56 in 2.0 %, 0.70 in 2.5 %, 0.60 in 3.0 %, 0.48 in 3.75 %, and 0.48 in 5.0 % NaCl. The strains of phenotype 1 were able to grow between 0 and 23.5–24.0 % (4.02–4.11 M) NaCl and those of phenotype 2 between 0 and 25.5 % (4.36 M) NaCl.

Table 1. Profiles of sugar fermentation of the 11 new isolates

Fermentations were scored as: +, positive; –, negative; w, weakly positive (as defined in Methods). All 11 strains ferment D-glucose, D-fructose, D-galactose, D-mannose, D-cellobiose, lactose, maltose, melibiose, sucrose, D-trehalose, D-raffinose, D-mannitol, starch, methyl α -D-glucoside, D-salicin and sodium gluconate, but do not ferment adonitol, *myo*-inositol, dulcitol or D-sorbitol.

Carbon compound	Phenotype 1						Phenotype 2				
	M2-1	M2-2 ^T	M2-3	M2-4	M9-1	M13-1	M23-1 ^T	M23-2	M23-3	M23-4	M23-5
L-Arabinose	–	–	–	–	–	–	+	+	+	+	+
D-Arabinose	–	–	–	–	–	–	–	–	w	–	–
D-Ribose	+	+	+	+	+	–	+	+	+	+	+
D-Xylose	–	–	–	–	–	–	+	+	+	+	+
D-Rhamnose	–	–	–	–	–	–	–	–	w	–	–
D-Melezitose	–	–	–	–	–	–	+	+	+	+	+
Glycerol	+	+	+	+	+	+	w	w	w	w	w
Inulin	–	–	–	–	–	–	+	+	+	+	+

The isolates were thus slightly halophilic (Kushner, 1978; Kushner & Kamekura, 1988) and highly halotolerant. These characteristics are the physiological features common to most members of the HA group. Other than the present isolates, only a few taxa of lactic acid bacteria that are characteristically halophilic and highly halotolerant (able to grow at NaCl concentrations > 15 %) have been described to date: *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* isolated from salted foods (Iizuka & Yamasato, 1959; Satomi *et al.*, 1997), *Marinilactibacillus psychrotolerans* isolated from marine organisms (Ishikawa *et al.*, 2003b), and *Alkalibacterium olivapovlenticus* isolated from alkaline edible-olive wash-water (Ntougias & Russell, 2001). *Weissella halotolerans*, which was isolated from meat products, has a salt tolerance of 14 % (Kandler *et al.*, 1983).

The initial medium pH that resulted in optimum growth was between 8.0 and 9.0 for phenotype 1 and 9.5 for phenotype 2. No growth was observed in media for which the initial pH was ≤ 6.0 or ≥ 10.0 for phenotype 1, or ≤ 5.5 or ≥ 10.5 for phenotype 2. For strain M2-2^T, phenotype 1, the maximum specific growth rates (h^{-1}) were 0.38 at pH 7.0, 0.40 at pH 7.5, 0.42 at pH 8.0, 0.52 at pH 8.5, 0.50 at pH 9.0 and 0.14 at pH 9.5. For strain M23-1^T, phenotype 2, they were 0.46 at pH 7.0, 0.46 at pH 7.5, 0.46 at pH 8.0, 0.46 at pH 8.5, 0.48 at pH 9.0, 0.68 at pH 9.5 and 0.40 at pH 10.0. The final pH of cultures in 2.5 % NaCl GYPF broth reached 5.2–6.0, which was 0.5–1.3 pH units lower than the minimum pH required to initiate growth. According to Jones *et al.* (1994), alkaliphiles are organisms that grow optimally at pH values greater than 8. The isolates were alkaliphiles, as they grew optimally at pH values between 8.0 and 9.0 (phenotype 1) or 9.5 (phenotype 2).

Commonly occurring lactic acid bacteria are comparatively acid tolerant (their broth cultures attaining a final pH value < 3.0) and grow optimally at neutral to slightly acid pH, such as pH 6.0. Although the isolates produce acid, their growth optima lie considerably to the alkaline side, and the pH has to be > 6.0 for growth initiation, both of which are uncommon characteristics among typical lactic acid bacteria. *Marinilactibacillus psychrotolerans* (Ishikawa *et al.*, 2003b) and *Alkalibacterium olivapovlenticus* (Ntougias & Russell, 2001) are two other organisms that are not acid tolerant and not neutrophilic, but are alkaliphilic lactic acid bacteria. In the HA group, *Amphibacillus xylanus*, which was isolated from an alkaline compost of manure with grass and rice straw and has an energy metabolism conforming to that of the isolates (discussed later), is also alkaliphilic, growing optimally at pH 8.0–10.0 (Niimura *et al.*, 1990). *Pediococcus urinaeequi*, which was described as being isolated from horse urine, grows optimally at pH 8.5–9.0, as deduced from the amount of acid produced in buffered broth cultures that were started at different initial pH values (Nakagawa & Kitahara, 1959).

The strains reported here are marine inhabitants, as they were isolated from living and decaying marine organisms and had physiological properties consistent with the

physico-chemical conditions found in sea water [total salt concentration 3.2–3.8 % (w/v), pH 8.2–8.3 (surface)]. That is, they grow optimally in 2.0–3.0 % NaCl (and can grow in 0–25.5 % NaCl) and at pH 8.0–9.5 (and can grow at a pH range of 6.0–10.0). Isolation and taxonomic studies of lactic acid bacteria from marine environments are few to date and have generally been confined to isolates from cultured fish (Ringø & Gatesoupe, 1998; Gatesoupe, 1999). Recently, members of the genus *Marinilactibacillus* have been isolated: *Marinilactibacillus* sp. from coastal sub-seafloor sediment of the Okhotsk Sea (Inagaki *et al.*, 2003) and *Marinilactibacillus piezotolerans* from deep sub-seafloor sediment of the Nankai Trough (Toffin *et al.*, 2005). *Marinilactibacillus psychrotolerans*, which has been isolated from a decaying marine alga, a living sponge and a fresh shellfish, is a marine-inhabiting lactic acid bacterium with growth optima at 2.0–3.75 % NaCl and pH 8.5–9.0 (Ishikawa *et al.*, 2003b). For such organisms, Ishikawa *et al.* (2003b) proposed the term 'marine lactic acid bacteria'. Thus, the present isolates can be regarded as additional representatives of marine lactic acid bacteria on the basis of habitat, physiological properties and lactic acid fermentation (described later).

The optimum growth temperatures for the isolates were 30–37 °C (phenotype 1) and 37–40 °C (phenotype 2). The maximum specific growth rates (h^{-1}) of strain M2-2^T, phenotype 1, were 0.48 at 25 °C, 0.58 at 30 °C, 0.60 at 37 °C, 0.42 at 40 °C and 0.06 at 42.5 °C. Those of strain M23-1^T, phenotype 2, were 0.62 at 25 °C, 0.64 at 30 °C, 0.74 at 37 °C, 0.74 at 40 °C and 0.18 at 42.5 °C. Growth occurred between 5–10 and 40 °C and between 5 (–1.8 °C for strain M23-3) and 45 °C for phenotypes 1 and 2, respectively.

The fermentation products of glucose and the effects of the initial pH of the fermentation medium were investigated. Under anaerobic cultivation in 2.5 % NaCl GYPF broth, the isolates produced lactate in yields of 50–60 % of the amount of glucose consumed, depending on the isolate. The other end-products were formate, acetate and ethanol in a molar ratio of approximately 2 : 1 : 1. No gas was produced. The L-isomer of lactate was 80–95 % of the total lactate produced. The amount of lactate relative to that of the other three products was markedly affected by the initial pH of the fermentation medium. For the representative selected strains M2-2^T (phenotype 1) and M23-1^T (phenotype 2), the products from glucose in relation to the pH of the medium were determined by using buffered media. The lactate yield relative to glucose consumed by strain M2-2^T at pH 8.0, 57 %, increased to 75 % at the more acidic pH 7.0, whereas it decreased remarkably to 22 % at the more alkaline pH 9.0; that of strain M23-1^T at suboptimum pH 9.0, 37 %, increased at more acidic pH values, 57 % at pH 8.0 and 65 % at pH 7.0 (Table 2). For each of the initial cultivation pH values, the drop in pH value at the point of product analysis was ≤ 0.5 pH units. At all pH values, carbon recovery from glucose consumed was about 100 %, and the molar ratios of formate, acetate and ethanol produced were generally approximately 2 : 1 : 1 (Table 2).

Table 2. Effect of initial pH of the culture medium on the product balance of glucose fermentation by the representative strains M2-2^T and M23-1^T

Strain	End-products [mol (mol glucose) ⁻¹]				Lactate yield from consumed glucose (%)	Carbon recovery (%)
	Lactate	Formate	Acetate	Ethanol		
M2-2^T						
pH 7	1.50	0.73	0.27	0.16	75	101
pH 8	1.13	0.81	0.37	0.46	57	98
pH 9	0.45	1.84	0.74	0.47	22	93
M23-1^T						
pH 7	1.30	0.76	0.28	0.18	65	93
pH 8	1.13	0.81	0.45	0.53	57	103
pH 9	0.73	1.28	0.51	0.32	37	86
<i>Marinilactibacillus psychrotolerans</i>						
M13-2^T*						
pH 7	2.02	0.15	0.04	0.05	101	107
pH 8	1.50	0.52	0.20	0.19	75	97
pH 9	1.29	0.81	0.35	0.20	66	98

*Data from Ishikawa *et al.* (2003b).

The similar alkaliphilic lactic acid bacteria *Marinilactibacillus psychrotolerans* and *Alkalibacterium olivapovliticus* likewise produce formate, acetate and ethanol in addition to lactate under anaerobic conditions, and their product ratios were similarly affected by the initial pH of the fermentation medium (Ishikawa *et al.*, 2003b). Also, in the lactate-producing facultative anaerobes *Exiguobacterium aurantiacum* (which lies in the phylogenetic radiation of 'classical' *Bacillus*) and *Paraliobacillus ryukyuensis* (which belongs to the HA group), glucose fermentation responded similarly to pH changes in the cultivation medium (Gee *et al.*, 1980; Collins *et al.*, 1983; Ishikawa *et al.*, 2002), as for the present isolates. Alternation of product composition in glucose fermentation (i.e., decreased lactate production and increased production of the other three products) at alkaline pH and at limited glucose concentrations has also been reported for homofermentative lactic acid bacteria such as *Streptococcus liquefaciens* (*Enterococcus faecalis*), *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus bulgaricus* (*Lactobacillus delbrueckii* subsp. *bulgaricus*) (Gunsalus & Niven, 1942; Carlsson & Griffith, 1974; Rhee & Pack, 1980). In these bacteria, pyruvate is converted to lactate by lactate dehydrogenase and to formate, acetate and ethanol by pyruvate-formate lyase at a molar ratio of 2:1:1: the product balance depends on the relative activities of the two enzymes involved (Carlsson & Griffith, 1974; Yamada & Carlsson, 1975; Axelsson, 1993). Kandler & Weiss (1986) classified lactic acid fermentation in the genus *Lactobacillus* into three groups: group I, obligately homofermentative lactobacilli; group II, facultatively heterofermentative lactobacilli; and group III, obligately heterofermentative lactobacilli. Whereas *Lactobacillus* species belonging to group II produce lactate almost exclusively from hexose under their usual growth conditions, they produce lactate, formate, acetate and

ethanol under glucose limitation (Kandler & Weiss, 1986). It is noteworthy that the present isolates, as well as *Marinilactibacillus psychrotolerans* and *Alkalibacterium olivapovliticus*, are lactic acid bacteria in which pyruvate-formate lyase would be active to produce formate, acetate and ethanol under their normal growth conditions.

Products from glucose under aerobic cultivation conditions were acetate, pyruvate and lactate, but formate and ethanol were not produced by the isolates or *Marinilactibacillus psychrotolerans* M13-2^T (see Supplementary Table S1 in IJSEM Online). Carbon recovery was not balanced. This imbalance can be ascribed to CO₂ generation, if the metabolism of *Amphibacillus xylanus*, another facultatively anaerobic bacterium belonging to the HA group (Niimura *et al.*, 1990), is relevant. *Amphibacillus xylanus* lacks catalase, cytochromes and quinones. This bacterium has both an anaerobic pathway for glucose metabolism and an oxidative metabolic pathway that is not mediated by the respiratory chain (Niimura *et al.*, 1989). Under anaerobic conditions, the end-products from glucose are formate, acetate and ethanol in a molar ratio of 2:1:1. Acetyl CoA and formate are formed from pyruvate, mediated by pyruvate-formate lyase. NADH produced through glycolysis is regenerated to NAD by aldehyde dehydrogenase and alcohol dehydrogenase. Under aerobic conditions, acetate and CO₂ are the end-products from glucose. Coupled with the conversion of pyruvate to acetyl CoA, NAD is reduced to NADH by pyruvate dehydrogenase and CO₂ is produced. NADH produced through glycolysis and pyruvate oxidation is regenerated to NAD by the NADH oxidase/peroxidase system, using O₂ as an electron acceptor. Consequently, ethanol production with generation of NADH does not occur under aerobic conditions. If we assume that

equimolar production of acetate and CO₂ also occurs in the isolates and in *Marinilactibacillus psychrotolerans*, carbon recovery under aerobic conditions can be calculated as 93–97 %.

The existence of the same oxidative pathway as that of *Amphibacillus xylanus* has been reported in several other species of lactic acid bacteria. For example, *S. mutans* has an oxidative pathway that is mediated by the NADH oxidase system (Fukui *et al.*, 1988). Liu *et al.* (2002) concluded that *Trichococcus flocculiformis*, which lacks cytochromes, also has this oxidative pathway, on the basis of an analysis of its metabolites and ATP production under air. Sakamoto & Komagata (1996) also reported NADH oxidase and NADH peroxidase activities and acetate production under aerobic conditions in the homofermentative lactic acid bacteria *Lactobacillus*, *Pediococcus* and *Streptococcus*, and in the heterofermentative lactic acid bacteria *Leuconostoc*. The isolates and *Amphibacillus xylanus* belong to the HA group in the phyletic radiation of 'classical' *Bacillus*, and *Marinilactibacillus psychrotolerans* and the lactic acid bacteria mentioned here belong to the radiation in which the usual lactic acid bacteria are located. All of these may have an identical oxidative metabolic pathway, even though they are located in different phyletic radiations. Thus, this metabolic system might be a common feature of aerotolerant fermentative bacteria.

The isolates possessed *meso*-diaminopimelic acid in the cell-wall peptidoglycan, as demonstrated by TLC. The peptidoglycan type of the genera belonging to the HA group is type A1 γ , *meso*-diaminopimelic acid direct linkages, except for *Halobacillus* species and *Filobacillus* species. The cellular fatty acid compositions of the representative strains M2-2^T (phenotype 1) and M23-1^T (phenotype 2) were characterized by straight-chain, anteiso-branched saturated, iso-branched saturated and monounsaturated acids (see Supplementary Table S2 in IJSEM Online). The major cellular fatty acids were a-C_{13:0} and C_{16:0}. Among the members of the HA group and other 'classical' *Bacillus* species that possess anteiso fatty acids as common features, possession of a-C_{13:0} as a major component is characteristic of the isolates (see Supplementary Table S2 in IJSEM Online).

Respiratory quinones and cytochromes were not present in strains M2-2^T and M23-1^T. Along with *Amphibacillus xylanus*, the absence of these respiratory components is characteristic among members of the HA group, which is composed of many aerobic and facultatively anaerobic genera and species.

The G+C contents of the DNA of the new isolates fell into narrow ranges: 39.6–40.7 mol% for phenotype 1 and 38.5–40.0 mol% for phenotype 2 (Table 3). The DNA–DNA relatedness values among the strains of phenotype 1

Table 3. DNA base composition and DNA–DNA relatedness among the new isolates and related taxa

Strain	DNA base composition (mol% G+C)*	Relative binding (%) of DNA from:	
		M2-2 ^T	M23-1 ^T
Phenotype 1			
M2-1	39.6	84	ND
M2-2 ^T	40.2	100	35
M2-3	40.0	82	ND
M2-4	40.2	87	ND
M9-1	40.7	92	21
M13-1	40.5	85	29
Phenotype 2			
M23-1 ^T	38.5	41	100
M23-2	39.2	ND	96
M23-3	39.7	ND	79
M23-4	38.7	ND	82
M23-5	40.0	ND	83
<i>Paraliobacillus ryukyuensis</i> O15-7 ^T	35.6 ^a	4	4
<i>Amphibacillus xylanus</i> JCM 7361 ^T	36.0 ^b	5	4
<i>Amphibacillus fermentum</i> DSM 13869 ^T	41.5 ^c	22	14
<i>Amphibacillus tropicus</i> DSM 13870 ^T	39.2 ^c	24	21
<i>G. halotolerans</i> DSM 11805 ^T	39.4 ^d	3	2
<i>G. dipsosauri</i> DSM 11125 ^T	35.6 ^e	4	4

ND, Not determined.

*Data from: *a*, Ishikawa *et al.* (2002); *b*, Niimura *et al.* (1990); *c*, Zhilina *et al.* (2001); *d*, Wainø *et al.* (1999); *e*, Lawson *et al.* (1996).

were 82–92 % and those among the strains of phenotype 2 were 79–96 %. Isolates M2-2^T, M9-1 and M13-1 of phenotype 1 showed low DNA–DNA relatedness values (35, 21 and 29 %, respectively) to M23-1^T from phenotype 2, and M23-1^T from phenotype 2 also showed low DNA–DNA relatedness (41 %) to M2-2^T from phenotype 1 (Table 3). On the basis of their DNA–DNA relatedness, the isolates constitute two genomic species; furthermore, the strains within each genomic species correspond to the two phenotypes that were determined from the sugar fermentation profiles (Table 1). Levels of DNA–DNA relatedness between the two type strains and strains of the phylogenetically related genera *Amphibacillus*, *Gracilibacillus* and *Paraliobacillus* were 2–24 %, as low as levels between distinct species (Table 3).

Complete sequences of the 16S rRNA gene, 1491 bases in length and covering positions 41–1508 (*Escherichia coli* numbering system; Brosius *et al.*, 1978), were determined for the type strain of each phenotype. The sequences were aligned and compared with the sequences of 29 species of related

bacteria and lactic acid bacteria from public databases. The 16S rRNA gene sequence similarity value between M2-2^T (phenotype 1) and M23-1^T (phenotype 2) was 99.1 %. Pairwise analysis revealed that the new isolates exhibited the highest similarity values to the genera *Paraliobacillus* (94.8–95.1 % similarity), *Amphibacillus* (92.9–94.3 %), *Gracilibacillus* (93.7–94.1 %) and *Virgibacillus marismortui* (93.8–94.2 %). A phylogenetic tree constructed by using the neighbour-joining method showed that the two phenotypes constitute an independent line of descent within the HA group in rRNA group 1 of the phyletic group classically defined as the genus *Bacillus*, and occupy a phylogenetic position that is closely related to the genera *Paraliobacillus*, *Gracilibacillus* and *Amphibacillus* (Fig. 2). This relationship between the isolates and these three genera was also found in the tree constructed using the maximum-likelihood algorithm (see Supplementary Fig. S1 in IJSEM Online).

The isolates possess all the essential characteristics of lactic acid bacteria that have been attributed to the most typical lactic acid bacteria, including production of lactic acid

Fig. 2. Phylogenetic relationships between the new isolates and some other related bacteria, based on 16S rRNA gene sequences. *Exiguobacterium aurantiacum* NCDO 2321^T was used as an outgroup. The tree, constructed by using the neighbour-joining method, is based on comparison of approximately 1380 nucleotides. Bar, 0.01 K_{nuc} . Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points; only values above 50 % are shown. *Bacillus* rRNA group 1 according to Ash *et al.* (1991) is shown.

through the Embden–Meyerhof pathway and lack of catalase, quinones, cytochromes and respiratory metabolism, but are novel lactic acid bacteria in terms of phylogeny. Typical lactic acid bacteria can be considered to have evolved retrogressively from facultative anaerobes as close ancestors. This assumption is supported by several findings. For example, *Streptococcus faecalis* (*Enterococcus faecalis*) has a haem-dependent cytochrome (Whittenbury, 1964; Bryan-Jones & Whittenbury, 1969). Whittenbury (1964) argued that its haem-dependent cytochrome may be a rudimentary respiratory system. In several lactic acid bacteria, enzymes involved in the TCA cycle, cytochromes, haem-dependent catalase and quinones, have been found (Whittenbury, 1964; Pritchard & Wimpenny, 1978; Morishita *et al.*, 1999; Wang *et al.*, 2000). The present isolates also could have evolved to lactic acid bacteria by following independent but similar evolutionary processes within the HA group, while retaining physiological characteristics consistent with the physico-chemical factors of salt concentration and pH that prevail in marine environments. This hypothetical consideration is supported by the close relationship between the new isolates and, on the one hand, *Paraliobacillus ryukyuensis*, which has catalase, quinones and cytochromes but requires sugars for growth under both aerobic and anaerobic conditions and performs lactic acid fermentation, and, on the other, *Amphibacillus xylanus*, which lacks catalase, quinones and cytochromes and produces formate, acetate and ethanol via the Embden–Meyerhof pathway. The HA group should be an interesting phylogenetic group for studying the possible evolution of a common ancestor to divergent forms, as it is a compact cluster and includes aerobes, facultative anaerobes and lactic acid bacteria, rods and cocci, and has various behaviours in relation to salinity and pH.

The present strains that were isolated from decaying algae and a living sponge are marine-inhabiting lactic acid bacteria that are slightly halophilic, extremely halotolerant and alkaliphilic. The isolates constitute an independent phylogenetic lineage within the HA group, which is in rRNA group 1, one of the phyletic groups classically defined as the genus *Bacillus*. The HA group is composed of 11 genera, most of which inhabit saline to hypersaline environments. Each of the genera or species is characterized by diverse features: oxidative and/or fermentative metabolism, bacilli or cocci in cellular morphology, spore formation and combinations of halophilic, halotolerant, alkaliphilic and alkalitolerant properties. The isolates differ from all members of the HA group in that they carry out aerobic and anaerobic energy metabolism (except for *Virgibacillus*, *Paraliobacillus* and *Amphibacillus*, which ferment sugars). Similar to *Paraliobacillus* and *Amphibacillus*, the present isolates also differ in that they require glucose (carbohydrates and related compounds) for growth even under aerobic conditions. They can be distinguished from the genus *Virgibacillus* by their lack of catalase and quinone and by their fatty acid compositions and from the genus *Paraliobacillus* by their lack of catalase, quinones and cytochromes (the presence of

cytochromes in *Paraliobacillus* was confirmed in this study). Although they share the properties of lack of catalase, cytochrome and quinone, and comparable energy metabolism with *Amphibacillus xylanus*, they can be distinguished from it by their fatty acid compositions, lack of spore formation and the effects of salt concentration and pH on growth.

The isolates were distinguished from all members of the HA group by their combination of morphological, physiological, biochemical and chemotaxonomic features (Table 4). The isolates conformed to two genera in the group of typical lactic acid bacteria, *Marinilactibacillus* and *Alkalibacterium*, with respect to the phenotypic properties of cellular morphology, motility, halophilic and halotolerant properties and lactic acid fermentation pattern. However, they could be distinguished from these genera by the chemotaxonomic characteristics of peptidoglycan type and cellular fatty acid composition. The isolates could be distinguished from other homofermentative lactic acid rods (*Lactobacillus*, *Carnobacterium* and *Paralactobacillus*), depending on the species, by motility, cellular fatty acid composition, DNA base composition and halophilic and alkaliphilic properties. In conclusion, on the basis of the phenotypic features and phylogenetic independence described above, the novel isolates should be classified as a new genus composed of two novel species. We propose the name *Halolactibacillus halophilus* gen. nov., sp. nov. for phenotype 1 as the type species of this genus and *Halolactibacillus miurensis* sp. nov. for phenotype 2.

Description of *Halolactibacillus* gen. nov.

Halolactibacillus [Ha.lo.lac'ti.ba.cil'lus. Gr. n. *hals* salt (loving); L. n. *lac lactis* milk; L. masc. n. *bacillus* stick, a small rod; N.L. masc. n. *Halolactibacillus* salt (loving) lactic acid rodlet].

Cells are Gram-positive, non-sporulating, straight rods, occurring singly, in pairs or in short chains, and elongated. Motile with peritrichous flagella. Catalase- and oxidase-negative. Negative for nitrate reduction, production of ammonia from L-arginine, production of dextran from sucrose and DNase. Weakly hydrolyse starch. Do not hydrolyse casein. Growth does not occur in the absence of sugars. Slightly halophilic and highly halotolerant; the optimum NaCl concentration for growth is 2–3 % (w/v), with a range of 0–25.5 % (w/v). Alkaliphilic; the optimum pH for growth is 8.0–9.5, with a range of 6.0–10.0. Growth occurs between 5–10 and 40.0–45.0 °C, with an optimum at 30–40 °C. In anaerobic cultivation, L-lactic acid is the major end-product from glucose. Considerable amounts of formate, acetate and ethanol are produced in a molar ratio of approximately 2:1:1, without gas production. Carbohydrates and related compounds are aerobically metabolized to acetate and pyruvate, as well as lactate. The cell-wall peptidoglycan is *meso*-diaminopimelic acid. Major cellular fatty acids are a-C_{13:0} and C_{16:0}. Respiratory quinones and cytochromes are absent. The G + C content of the DNA is

Table 4. Characteristics that distinguish the isolates from other members of the HA group in *Bacillus* rRNA group 1, *M. psychrotolerans* and *Alkalibacterium olivapovlenticus*

Taxa: 1, *Halolactibacillus* gen. nov.; 2, *Paraliobacillus ryukyuensis* (data from Ishikawa *et al.*, 2002); 3, *G. halotolerans* (Wainø *et al.*, 1999); 4, *G. dipsosauri* (Deutch, 1994; Lawson *et al.*, 1996; Wainø *et al.*, 1999); 5, *Amphibacillus xylanus* (Niimura *et al.*, 1990); 6, *Amphibacillus fermentum* (Zhilina *et al.*, 2001); 7, *Amphibacillus tropicus* (Zhilina *et al.*, 2001); 8, *Virgibacillus pantothenicus* (Heyndrickx *et al.*, 1998, 1999); 9, *Halobacillus halophilus* (Claus *et al.*, 1983; Spring *et al.*, 1996); 10, *Marinococcus albus* (Hao *et al.*, 1984); 11, *Oceanobacillus iheyensis* (Lu *et al.*, 2001); 12, *Filobacillus milosensis* (Schlesner *et al.*, 2001); 13, *Lentibacillus salicampi* (Yoon *et al.*, 2002); 14, *Cerasibacillus quisquiliarum* (Nakamura *et al.*, 2004); 15, *Marinilactibacillus psychrotolerans* (Ishikawa *et al.*, 2003b); 16, *Alkalibacterium olivapovlenticus* (Ntougias & Russell, 2001; Ishikawa *et al.*, 2003b). +, Positive; –, negative; ND, no data; ANR, anaerobic respiration; Asp, aspartic acid; F, fermentation; Glu, glutamic acid; *m*-Dpm, *meso*-diaminopimelic acid; Orn, ornithine.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Cell morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Cocci	Cocci	Rods	Rods	Rods	Rods	Rods	Rods
Spore formation	–	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–
Anaerobic growth	+	+	–	+	+	+	+	+	–	–	–	–	–	–	+	+
Catalase	–	+	+	+	–	+	+	+	+	+	+	+	+	+	–	–
Glucose requirement in aerobic cultivation	+	+	–	–	+	+	+	–	–	–	–	–	–	–	ND	ND
NaCl (%):																
Range	0–25.5	0–22	0–20	0–18.6 (KCl)	<6	1.0–19.7	1.0–20.9	0 to ≥10	2–15	5–20	0–21	2–23	2–23	<10	0–20	3–15
Opt.	2–3	0.75–3	0	3.7 (KCl)	ND	10.8	5.4–10.8	4	ND	5–15	3	8–14	4–8	0.5	2.0–3.75	3–5‡
pH:																
Range	6–10	5.5–9.5	5–10	6 to ≥10	8–10	7–10.5	8.5–11.5	ND	7–9	ND	6.5–10	6.5–8.9	>5	7.5–10	6.0–10.0	8.5–10.8
Opt.	8–9.5	7–8.5	7.5	7.5	ND	8.5–9	9.5–9.7	7	7.8	ND	7–9.5	7.3–7.8	6–8	8–9	8.5–9.0	9.0–10.2
Major isoprenoid quinones	None	MK-7	MK-7	MK-7	None	ND	ND	MK-7	MK-7	MK-7	MK-7	ND	MK-7	MK-7	None	None
Peptidoglycan type	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	ND	ND	<i>m</i> -Dpm	Orn-D-Asp	<i>m</i> -Dpm	ND	Orn-D-Glu	<i>m</i> -Dpm	<i>m</i> -Dpm	Orn-D-Glu	Orn-D-Asp
G + C content (mol%)	38.5–40.7	35.6	38	39.4	36–38	41.5	39.2	38.3	40.1–40.9	43.9–46.6	35.8	35	44	35.5	34.6–36.2	39.7 ± 1.0
Major cellular fatty acids	<i>a</i> -C _{13:0} C _{16:0}	ND	<i>a</i> -C _{15:0} C _{16:0} <i>a</i> -C _{17:0}	<i>i</i> -C _{15:0} <i>a</i> -C _{15:0} C _{16:0} <i>a</i> -C _{17:0}	<i>a</i> -C _{15:0} C _{16:0} <i>i</i> -C _{16:0}	ND	ND	<i>i</i> -C _{15:0} <i>a</i> -C _{15:0} <i>a</i> -C _{17:0}	ND	<i>a</i> -C _{15:0} <i>a</i> -C _{17:0}	<i>i</i> -C _{15:0} <i>a</i> -C _{15:0} <i>i</i> -C _{14:0}	ND	<i>a</i> -C _{15:0} <i>i</i> -C _{16:0}	<i>i</i> -C _{15:0}	C _{16:0} C _{16:1} Δ9 C _{18:1} Δ9	C _{16:0} C _{16:1} Δ7
Isolation source	Decaying marine algae, living sponge	Decaying marine alga	Surface mud, Great Salt Lake	Nasal salt glands of a desert iguana	Alkaline manure with grass and rice straw	Sediment, soda lake	Sediment, soda lake	Soils	Salt marsh soils	Solar saltern	Mud, Iheya Ridge	Beach sediment	Salt field	Decomposing system of kitchen refuse	Decaying marine algae, living sponge	Wash-waters of edible olives

*Spore formation was not observed but culture survived heating.

†Produced in aerobic cultivation.

‡Optimum for the type strain.

38.5–40.7 mol%. The type species is *Halolactibacillus halophilus*. As determined by 16S rRNA gene sequence analysis, the genus *Halolactibacillus* is located within the phylogenetic group composed of halophilic/halotolerant/alkaliphilic and/or alkalitolerant species in *Bacillus* rRNA group 1.

Description of *Halolactibacillus halophilus* sp. nov.

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

This species has all the characteristics that define the genus. In addition, it has the characteristics described below. Deep colonies in 2.5 % NaCl glucose-yeast extract-peptone-fish extract agar medium are pale-yellow and lenticular, with diameters of 2–4 mm after 3 days at 30 °C. Surface colonies are round, convex, entire, pale-yellow and transparent, with diameters of 0.8–1.0 mm after 3 days at 30 °C. Cells are 0.6–0.9 × 3.6–4.5 µm, occurring singly, in pairs or in short chains. The optimum NaCl concentration for growth is 2.0–3.0 %, with a range of 0 to 23.5–24.0 %. The optimum pH for growth is 8.0–9.0, with a range of 6.5–9.5. Growth occurs between 5–10 and 40 °C, with an optimum at 30–37 °C. Lactic acid is the major fermentation product from glucose: 40–50 % of glucose consumed is converted to formate, acetate and ethanol. Lactate yield decreases at higher pH of the fermentation medium. The following carbohydrates and related compounds are fermented: D-ribose, D-glucose, D-fructose, D-galactose, D-mannose, D-cellobiose, lactose, maltose, melibiose, sucrose, D-raffinose, D-salicin, D-trehalose, D-melezitose, D-mannitol, methyl α-D-glucoside, glycerol, starch and sodium gluconate. L-Arabinose, D-arabinose, D-xylose, D-rhamnose, D-melezitose, D-sorbitol, dulcitol, *myo*-inositol, adonitol and inulin are not fermented. The G + C content of the DNA is 39.6–40.7 mol%.

The type strain is M2-2^T (=DSM 17073^T = IAM 15242^T = NBRC 100868^T = NRIC 0628^T). Isolated from decaying marine algae and a living sponge collected at Oura beach, Miura Peninsula, Kanagawa Prefecture, Japan. The G + C content of the type strain is 40.2 mol%.

Description of *Halolactibacillus miurensis* sp. nov.

Halolactibacillus miurensis (mi.u.ren'sis. N.L. masc. adj. *miurensis* from the Miura Peninsula, Japan, where the strains were isolated).

This species has all the characteristics that define the genus. In addition, it has the characteristics described below. Deep colonies in 2.5 % NaCl glucose-yeast extract-peptone-fish extract agar medium are pale-yellow and lenticular, with diameters of 2–4 mm after 3 days at 30 °C. Surface colonies are round, convex, entire, pale-yellow and transparent, with diameters of 1.0–1.5 mm after 3 days at 30 °C. Cells are 0.6–0.9 × 3.6–4.5 µm, occurring singly, in pairs or short chains. The optimum NaCl concentration for growth is 2.5–3.0 % (w/v), with a range of 0–25.5 %. The optimum

pH for growth is 9.5, with a range of 6.0–6.5 to 10.0. Growth occurs at 5–45 °C, with an optimum at 37–40 °C. Lactic acid is the major fermentation product from glucose: 40–50 % of glucose consumed is converted to formate, acetate and ethanol. Lactate yield decreases at higher pH of the fermentation medium. The following carbohydrates and related compounds are fermented: L-arabinose, D-ribose, D-xylose, D-glucose, D-fructose, D-galactose, D-mannose, D-cellobiose, lactose, maltose, melibiose, sucrose, D-raffinose, D-salicin, D-trehalose, D-melezitose, D-mannitol, methyl α-D-glucoside, glycerol (weak), inulin, starch and sodium gluconate. D-Arabinose, D-rhamnose, D-sorbitol, dulcitol, *myo*-inositol and adonitol are not fermented. The G + C content of the DNA is 38.5–40.0 mol%.

The type strain is strain M23-1^T (=DSM 17074^T = IAM 15247^T = NBRC 100873^T = NRIC 0633^T). Isolated from decaying alga at Oura beach, Miura Peninsula, Kanagawa Prefecture, Japan. The G + C content of the type strain is 38.5 mol%.

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