

Marinilactibacillus psychrotolerans gen. nov., sp. nov., a halophilic and alkaliphilic marine lactic acid bacterium isolated from marine organisms in temperate and subtropical areas of Japan

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A novel marine lactic acid rod bacterium has been described for eight strains isolated from living and decomposing marine organisms collected from temperate and subtropical areas of Japan. The isolates were Gram-positive, catalase-negative, non-sporulating and motile with peritrichous flagella. They were slightly halophilic, highly halotolerant and alkaliphilic; the optimum NaCl concentration for growth was 2.0–3.75 % (w/v) with a range from 0 to 17.0–20.5 % (depending on the strain); the optimum pH was between 8.0 and 9.5 with a range from 6.0 to 10.0. They were psychrotolerant, growing well at -1.8°C with a maximum at $40\text{--}45^{\circ}\text{C}$ and the optimum at $37\text{--}40^{\circ}\text{C}$. Lactate yields were 87–100 % per consumed glucose; the residual products were formate, acetate and ethanol with a molar ratio of approximately 2 : 1 : 1. The product composition was markedly affected by the pH of fermentation medium; at higher pH, the yield of lactate decreased (60–65 % at pH 9.0) and that of other products increased conversely. The cell-wall peptidoglycan type was type A4 β , Orn-D-Glu, whereas that of the genus *Alkalibacterium*, the phylogenetically closest lactic acid bacterium, was type A4 β , Orn-D-Asp. The major cellular fatty acids were C16:0, C16:1 Δ 9, C18:0 and C18:1 Δ 9 (oleic acid). The G + C content of the DNA was 34.6–36.2 mol%. The eight isolates were phenotypically homogeneous and formed a single genomic species. The 16S rRNA gene sequence analysis indicated that the isolates constituted an independent phylogenetic lineage within the radiation of lactic acid bacteria with 96.2 % similarity to the genus *Alkalibacterium*. The secondary structure and the nucleotide sequence of the V6 region of the 16S rRNA were characteristic of the organism among other related lactic acid genera. On the bases of phenotypic and phylogenetic distinctness, the organism was proposed to belong to a new genus and species, *Marinilactibacillus psychrotolerans* gen. nov., sp. nov. The type strain, M13-2^T (G + C = 36.2 mol%), has been deposited in the IAM, NBRC, NCIMB and NRIC culture collections as IAM 14980^T, NBRC 100002^T, NCIMB 13873^T and NRIC 0510^T, respectively.

INTRODUCTION

Marine environments are characterized by the presence of salts (total salts concentration 3.2–3.8 %, w/v) and by an alkaline reaction (surface water pH of 8.2–8.3) derived from the physico-chemical nature of seawater, both of which affect the way of life of organisms inhabiting such

conditions. The sea, especially in coastal areas, is abundant in biota, dead or living, which are rich sources of nutrients necessary for the growth of heterotrophic micro-organisms. Many ecological and taxonomic studies on marine bacteria have been carried out, but most have been concerned with aerobic or facultatively anaerobic, Gram-negative bacteria. Isolation and taxonomic studies of lactic acid bacteria from marine environments to date are few and have generally been confined to those from cultured fish (Ringø & Gatesoupe, 1998; Gatesoupe, 1999). Franzmann *et al.* (1991) isolated two species of lactic acid bacteria, *Carnobacterium funditum* and *Carnobacterium alterfunditum*, from the water of Ace Lake in Antarctica of possible seawater origin (Masuda *et al.*, 1988).

Published online ahead of print on 1 November 2002 as DOI 10.1099/ijs.0.02446-0.

The DDBJ accession numbers for the 16S rRNA gene sequences of strains M13-2^T, M13-3, M13-4, M13-5, M13-6, M13-7, O1-1, O21 and *Carnobacterium mobile* DSM 4848^T are AB083406, AB083407, AB083408, AB083409, AB083410, AB083411, AB083412, AB083413 and AB083414, respectively.

In this paper, we describe the isolation (from dead or living marine organisms), taxonomic characterization and phylogenetic position of a novel halophilic and alkaliphilic lactic acid bacterium, for which the name *Marinilactibacillus psychrotolerans* gen. nov., sp. nov. is proposed. We show further that the obligately alkaliphilic *Alkalibacterium olivapovlenticus* (Ntougias & Russell, 2001), corrected on notification to *Alkalibacterium olivapovlenticus* (International Committee on Systematics of Prokaryotes, 2001), the closest phylogenetic neighbour of the new isolates, is a member of the lactic acid bacteria.

METHODS

Samples for isolation of lactic acid bacteria. The samples for the isolation of lactic acid bacteria were collected from Oura beach, Miura Peninsula, Kanagawa Prefecture, in the middle of the Japanese mainland, a temperate area, in July 1998, and from a fore-shore site near the Oujima Islet and a fish market in the city of Naha, both in Okinawa in the Southernmost part of Japan, a sub-tropical area, in September 1998. The collected samples were living, fresh or decomposing sponges, algae, shellfish, crabs and fish.

Isolation media. For the samples collected from the Miura Peninsula, a 7% NaCl GYPB isolation broth was used for the enrichment culture in the isolation procedure. It was composed of 10 g glucose, 5 g yeast extract (Oriental Yeast), 5 g Polypeptone (Nippon Seiyaku), 5 g beef extract (Difco), 1 g K_2HPO_4 , 70 g NaCl, 10 g sodium acetate, 0.5 g Tween 80, 10 mg cycloheximide, 10 mg colistin, 15 mg nalidixic acid, 20 mg monofluoroacetic acid, 10 mg sodium azide, 15 g Na_2CO_3 (as a buffer) and 10% (w/v) seawater to 1000 ml. The final pH was 10.0. The medium was sterilized by filtration through a membrane filter with a 0.2 µm pore size (Toyo Roshi Kaisha). The pour-plating medium was 7% NaCl GYPB isolation agar (1.3% agar) supplemented with 5 g $CaCO_3$, Na_2CO_3 and $CaCO_3$ were autoclaved separately at 121 °C for 15 min and added aseptically (final pH, 10.0). The agar medium for overlaying on the pour plates contained 0.1% (w/v) sodium thioglycolate and the inorganic ingredients of the 7% NaCl GYPB isolation broth. For the samples collected from Okinawa, two series of media were used for enrichment. One medium, the 7% NaCl GYPF isolation broth, was the same as the 7% NaCl GYPB isolation broth but with Extract Bonito (Wako Pure Chemical Industries) instead of beef extract, with sodium acetate and Tween 80 omitted, and with 5 ml of a salts solution added, and prepared with distilled water to 1000 ml, pH 7.5. The medium was sterilized by filtration. The salt solution was composed of (ml^{-1}): 40 mg $MgSO_4 \cdot 7H_2O$, 2 mg $MnSO_4 \cdot 4H_2O$ and 2 mg $FeSO_4 \cdot 7H_2O$ (Okada *et al.*, 1992). For pour-plating, the 7% NaCl GYPF isolation agar (1.3% agar) supplemented with 5 g $CaCO_3$ l^{-1} was used. The overlaying agar medium contained 0.1% (w/v) sodium thioglycolate and the inorganic ingredients of the 7% NaCl GYPF isolation broth. The other media for the enrichment were 12 and 18% (for a subsequent second enrichment) NaCl GYPFSK isolation broths, which consisted of 10 g glucose, 5 g yeast extract, 5 g Polypeptone, 5 g Extract Bonito, 50 ml soy sauce, 10 g K_2HPO_4 , 110/170 g NaCl, 1 g sodium thioglycolate, 5 ml salts solution, 10 mg cycloheximide and distilled water to 1000 ml. The medium was adjusted to pH 7.5 and autoclaved at 110 °C for 10 min. For pour-plating, the 12% NaCl GYPFSK isolation agar (2.0% agar, final pH 7.5) supplemented with 5 g $CaCO_3$ l^{-1} was used.

Isolation procedure and strains for study. For enrichment with the 7% NaCl GYPB isolation broth (Miura Peninsula samples) or the 7% NaCl GYPF isolation broth (Okinawa samples), small pieces

of the samples (intestinal contents or whole bodies for animal samples) were soaked in 5 ml enrichment medium immediately after collection. After incubation at 30 °C for 3 days, a portion of the enrichment culture broth, whose pH had decreased to below 7.0, was put into fresh medium and incubated anaerobically at 30 °C for 2 days. A portion of the second enrichment culture broth was pour-plated and a plate was overlaid with the overlaying agar medium. In another isolation series of the Okinawa samples, the 12% NaCl GYPFSK isolation broth was used for a first enrichment incubated for 21 days, and the 18% NaCl GYPFSK isolation broth was used for a second enrichment incubated for 15 days, both at 25 °C in standing culture. Pour-plated agar media were not overlaid. Lenticular colonies were picked up and the isolates were purified with repeated pour-plating. Six isolates, M13-2^T, M13-3, M13-4, M13-5, M13-6 and M13-7, were obtained from a living sponge from the Miura Peninsula. One isolate, O1-1, was from a raw Japanese ivory shell (*Babylonia japonica*) cultured with the 7% NaCl GYPF isolation broth enrichment, and another isolate, O21, was from a decomposing alga cultured with the 12–18% NaCl GYPFSK isolation broth enrichment. Both samples were collected from Okinawa. *Alkalibacterium olivapovlenticus* NCIMB 13710^T, *Carnobacterium divergens* IFO 15683^T and *Carnobacterium mobile* DSM 4848^T were used as reference strains. These were obtained from the National Collections of Industrial, Food and Marine Bacteria (NCIMB), Aberdeen, UK, the Institute for Fermentation, Osaka (IFO), Osaka, Japan and Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany, respectively.

Media and conditions for cultivation and taxonomic characterization. Unless otherwise stated, a 2.5% NaCl GYPF broth, composed of 10 g glucose, 5 g yeast extract, 5 g Polypeptone, 5 g Extract Bonito, 1 g K_2HPO_4 , 25 g NaCl, 1 g sodium thioglycolate, 5 ml salt solution and distilled water to 1000 ml, was used as the basal medium for cultivation and taxonomic characterization. The medium was adjusted to pH 8.5 and sterilized by filtration. In some experiments, a 2.5% NaCl GYPF broth with the concentration of K_2HPO_4 raised to 1% was used to improve growth by buffering the medium (2.5% NaCl GYPF broth). The medium used for the maintenance of cultures was prepared with 7% NaCl GYPF isolation agar by adding 12 g Na_2CO_3 and 3 g $NaHCO_3$ l^{-1} , omitting selective agents, and supplementing with $CaCO_3$. Main components, buffer compounds and $CaCO_3$ were sterilized separately. The final pH was 9.0. The isolates were stored at 5 °C and transferred at 1-month intervals.

Cultivation was at 30 °C unless otherwise stated. Anaerobic cultivation was conducted by absorbing the oxygen in a container for test tube cultures with AnaeroPack-Kenki (CO_2 -generated; Mitsubishi Gas Chemical). *Alkalibacterium olivapovlenticus* NCIMB 13710^T was cultivated and maintained in the same way as the new isolates. *Carnobacterium divergens* IFO 15683^T and *Carnobacterium mobile* DSM 4848^T were cultivated according to the usual methods.

Cultural and morphological characteristics. Cellular morphology was observed for unstained cells mounted on a 2% water agar film with phase-contrast microscopy. Cultural characteristics of colonies were observed with 2.5% NaCl GYPF agar (1.3% agar). Motility was examined microscopically for cells cultured in 2.5% NaCl GYPF broth and by observing the occurrence of diffused growth along the stab line in semi-solid 2.5% GYPF agar (0.15% agar). These two agar media were sterilized by autoclaving at 110 °C for 10 min. Gram staining was carried out by using a crystal violet/phenol solution (Merck) following the procedure as described by Murray *et al.* (1994). Spore formation was examined microscopically. Flagellation was observed in cells grown on 2.5% NaCl GYPF

agar incubated anaerobically and stained by the method of Nishizawa & Sugawara as described by the Alumni Association of The Institute of Medical Sciences, The University of Tokyo (1988).

Physiological and biochemical characteristics. The catalase and oxidase reactions were tested with cells grown in 2.5% NaCl GYPF broth. The oxidase activity was examined with an oxidase test strip (Eiken Chemical). To observe behaviour against oxygen concentration, 2.5% NaCl GYPFK soft agar (0.6% agar) in a tube was evenly inoculated with a portion of appropriately diluted young broth culture by mixing, and the density and size of the colonies that grew were observed. For the sugar fermentation tests, sugars were added to 2.5% NaCl GYPF broth without glucose in 1% concentrations and filter-sterilized. For the starch and inulin tests, the media were sterilized at 110 °C for 10 min. Production of acid was scored as positive when the titre of 0.1 M NaOH per 5 ml cultured broth was ≥ 0.25 ml and as weakly positive when it was ≥ 0.05 ml and < 0.25 ml. The catalase reaction, nitrate reduction, production of gas from glucose, production of ammonia from arginine, and gelatin (Difco; 2%, w/v) liquefaction tests were conducted by following Okada *et al.* (1992), using medium supplemented with 2.5% NaCl and adjusted to pH 8.5, or 2.5% NaCl GYPF medium.

Growth characteristics in relation to NaCl concentration, pH and temperature. The optimum NaCl concentration and range for growth were investigated with 2.5% NaCl GYPF broth prepared with NaCl concentrations from 0 to 3.0% (w/v) at 0.5% intervals, 3.75%, 5.0% and from 18.5 to 21.0% at 0.5% intervals. To determine maximum and minimum concentrations, growth during 7 days of observation was scored as positive when the titre of 0.1 M NaOH per 5 ml cultured broth was ≥ 0.25 ml and simultaneously, OD₆₆₀ was ≥ 0.1 . The effect of temperature on growth was investigated by incubating at -1.8 °C, from 0 to 40 °C at intervals of 5 °C, but with 37 °C replacing 35 °C, and from 40 to 47.5 °C at intervals of 2.5 °C. To determine maximum and minimum growth temperatures, growth was observed for 1 day to 3 weeks, depending on temperature, and scored as for the NaCl test. To study the effect of medium pH on growth, pH values of 2.5% NaCl GYPF broth were adjusted from 5.5 to 11.5 at intervals of 0.5 pH units. Maximum and minimum pHs were determined by measuring the pH of a non-inoculated medium exactly when growth occurred in a cultured broth with the same initial pH, because the pH of non-inoculated, alkaline medium gradually decreased during incubation. The occurrence of growth was judged by optical density as for the NaCl test. Growth optima for NaCl concentration, pH and temperature were determined by using the maximum specific growth rate, μ_{\max} (h⁻¹), calculated from growth curves given by OD₆₆₀.

Fermentation products. Fermentation products from glucose were analysed by HPLC for broth cultured for 4 days, and then centrifuged and filtered through an ultrafree-MC tube (0.2 µm pore size; Millipore) to remove cells. Organic acids were analysed by using an LC-10A HPLC system (Shimadzu) with a Shim-pack SCR-102N column (Shimadzu) or a Shodex OA HPLC system (Shodex) with a Shodex Ionpak KC-811. The amount of ethanol produced was estimated by using an LC-9A HPLC system (Shimadzu) with a Shim-pack SCR-101N column (Shimadzu). The amount of glucose was determined by the Somogyi method (Somogyi, 1945) or enzymically by using glucose oxidase (Wako Pure Chemical Industries). The isomeric forms of lactic acid produced were determined enzymically by using D- and L-lactic acid dehydrogenases (Roche Diagnostics).

Analysis of fermentation products formed at different pHs. Cultivation was performed in buffered 2.5% NaCl GYPF broth supplemented with 100 mM HEPPS and adjusted to different pH values. The pH was 7.0, 8.0 or 9.0 for strains M13-2^T and O21, and 8.0, 9.0 or 10.0 for *Alkalibacterium olivapovliticus* NCIMB 13710^T.

The analysis of fermentation products was conducted for cultured broth whose OD₆₆₀ had reached about 0.20 to minimize the effect of any decrease in pH during fermentation. Products were analysed by the same method as described above.

Fatty acid analysis. Cells were cultivated in 2.5% NaCl GYPFK broth. The freeze-dried cells were hydrolysed with 4 M HCl at 100 °C for 5 h (Kawahara *et al.*, 1979). The resultant fatty acids were extracted and methylated with a 5% HCl/methanol mixture at 100 °C for 3 h (Suto *et al.*, 1982). The fatty acid analysis was carried out according to the procedure as described by Goto *et al.* (2002). GC-MS was performed with a Hewlett Packard HP 5890 gas chromatograph equipped with a type DB-23 capillary column (J & B Scientific) and DX-303 and DA-5000 mass spectrometers (JEOL). The column temperature was programmed to increase from 50 to 130 °C at a rate of 70 °C min⁻¹ and from 130 to 210 °C at a rate of 3 °C min⁻¹. Fatty acid methyl esters were identified by comparing mass spectra and retention times with fatty acid methyl ester standards (Sigma or Supelco).

Detection of respiratory quinone. The lipid fraction was extracted from cells harvested from 2.5% NaCl GYPFK broth culture and freeze-dried as described by Sano *et al.* (1996). The presence of respiratory quinone was examined by TLC as described by Yamada & Kuraishi (1982).

Amino acid composition of the cell wall. Cells were cultivated in 2.5% NaCl GYPFK broth. The presence of diaminopimelic acid was examined by TLC by following the method of Hasegawa *et al.* (1983) as described by Okada *et al.* (1992). Peptidoglycan and its hydrolysate were prepared by the method of Komagata & Suzuki (1987). The amino acid composition was determined by using an LC-6A HPLC system (Shimadzu) with a Shim-pack Amino-Na ion exchange column (Shimadzu).

Determination of DNA base composition and DNA relatedness. To determine the DNA base composition and DNA relatedness, DNA was extracted and purified by following the combined methods of Marmur (1961) and Saito & Miura (1963). DNA base composition was determined by reverse-phase HPLC (Tamaoka & Komagata, 1984). DNA relatedness was estimated by the fluorometric DNA-DNA hybridization method in microdilution wells as described by Ezaki *et al.* (1989).

16S rRNA gene sequence determination. Cells were cultivated in 2.5% NaCl GYPFK broth. DNA was extracted and purified as described above. Gene fragments specific for the 16S rRNA-coding regions of the isolates were amplified by PCR with the following two primers: 20F (5'-AGTTTGATCATGGCTCA-3', positions 10–26) and 1540R (5'-AAGGAGGTGATCCAACCGCA-3', positions 1541–1521) (*Escherichia coli* numbering system; Brosius *et al.*, 1978) as described by Yanagi & Yamasato (1993). The amplified 16S rRNA gene was sequenced directly by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM model 310 genetic analyser (Perkin-Elmer). The following five primers were used: 20F, 1540R, 350F (5'-CCTACGGGAGGCAGCAGT-3', positions 341–358), 800F (5'-GTAGTCCACGCCGTAAACGA-3', positions 803–819) and 900R (5'-CGGCCGTACTCCCCAGGCGG-3', positions 898–879).

Phylogenetic analysis. Multiple alignment was performed by using the program CLUSTAL X (version 1.8; Thompson *et al.*, 1997). Percentage similarities among the new isolates and related organisms were calculated in pairs of homologous sequences of 1422–1468 bases. Phylogenetic distances (K_{nucl}) for the aligned sequences were calculated by using the 2-parameter method of Kimura (1980). The neighbour-joining method (Saitou & Nei, 1987) was used for constructing a phylogenetic tree. Hypervariable regions at positions 66–103 (including V1 region) and 1436–1457 (V5 region) were omitted from the

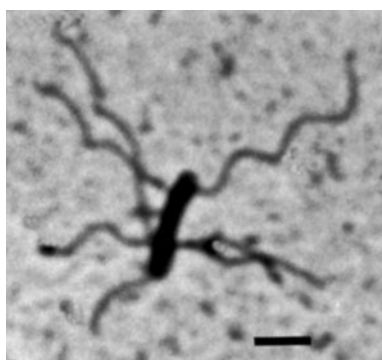


Fig. 1. Photomicrograph of cells and peritrichous flagella of *Marinilactibacillus psychrotolerans* M13-2^T grown anaerobically at 30 °C for 2 days on 2.5 % NaCl GYPFK agar. Bar, 2 µm.

phylogenetic analysis. The topology of the phylogenetic tree was evaluated by bootstrapping with 1000 replications (Felsenstein, 1985). Nucleotide sequences of the new isolates were deposited in the DNA Database of Japan (DDBJ). Accession numbers of the isolates deposited and the reference strains from the public databases are shown in Fig. 2.

RESULTS AND DISCUSSION

General taxonomic features

All eight newly isolated strains exhibited similar taxonomic features. The isolates were Gram-positive rods that were motile with peritrichous flagella (Fig. 1). Spores were not produced. Catalase was not produced by the cells cultivated in 2.5 % NaCl GYPF broth or in the medium with glucose

concentration reduced to 0.1 %. Growth was weak when the glucose concentration in 2.5 % NaCl GYPFK broth was reduced to 0.1 %, and no growth was seen when glucose was omitted. The isolates were able to grow, but with little vigour, on a 2.5 % NaCl GYPFK agar plate exposed to air. The density and size of colonies developed in semi-solid medium, evenly inoculated and incubated while exposed to air, was uniform from the surface to the bottom, and not affected by the oxygen concentration of the medium. A fairly wide range of carbohydrates, sugar alcohols and related carbon compounds was fermented with some variations among the isolates (Table 1). Cultural, morphological, physiological and biochemical features other than those described above are presented below and in the descriptions of the new genus and the new species.

Lactic acid fermentation and effect of cultivation pH on product composition

The end products of glucose fermentation by the different isolates were determined. Lactate was produced with yields, depending on isolates, of 87 to 100 % relative to the amount of consumed glucose, without gas production. The ratio of the L(+) isomer to total lactate produced was 75:94 %. Besides lactate, trace to small amounts of formate, acetate and ethanol were produced with a molar ratio of approximately 2:1:1.

The pH of the fermented medium had a large effect on the relative amount of lactate versus the other three products. For representative selected strains of M13-2^T and O21, fermentation products from glucose obtained by using buffered media are shown in Table 2. During the course of

Table 1. Sugar fermentation profiles of the eight new isolates

Fermentation is scored as: +, positive; −, negative; or W, weakly positive. All eight strains fermented D-ribose, D-xylose, D-fructose, D-glucose, D-mannose, D-cellobiose, maltose, sucrose, D-trehalose, D-mannitol, α-methyl-D-glucoside, D-salicin and sodium gluconate, but did not ferment *myo*-inositol.

Carbon compound	Strain							
	M13-2 ^T	M13-3	M13-4	M13-5	M13-6	M13-7	O1-1	O21
L-Arabinose	+	+	+	+	+	+	W	W
D-Arabinose	W	W	−	−	W	W	−	−
D-Galactose	+	+	+	+	+	+	+	−
D-Rhamnose	+	+	+	+	+	+	−	−
Lactose	W	W	W	W	W	W	+	+
Melibiose	+	−	+	−	−	−	−	−
D-Raffinose	−	−	+	−	−	−	+	W
D-Melezitose	−	−	+	−	−	−	−	W
Glycerol	W	W	W	W	W	W	+	+
Adonitol	−	−	−	−	−	−	−	W
Dulcitol	−	−	−	−	−	−	W	W
D-Sorbitol	−	−	−	−	−	−	+	W
Inulin	+	−	+	−	−	−	−	−
Starch	+	−	−	−	−	−	−	−

Table 2. Effect of pH on the balance of glucose fermentation by representative strains M13-2^T and O21, and *Alkalibacterium olivapovlriticus* NCIMB 13710^T

Strain	Initial pH	End products [mol (mol glucose) ⁻¹]				Lactate yield per consumed glucose (%)	Carbon recovery (%)
		Lactate	Formate	Acetate	Ethanol		
M13-2 ^T	7	2.02	0.15	0.04	0.05	101	107
	8	1.50	0.52	0.20	0.19	75	97
	9	1.29	0.81	0.35	0.20	66	98
O21	7	1.69	0.32	0.08	0.26	85	101
	8	1.56	0.63	0.22	0.32	78	107
	9	1.13	0.82	0.37	0.45	57	98
NCIMB 13710 ^T	8	1.35	0.97	0.44	0.52	67	116
	9	1.01	1.21	0.53	0.52	50	106
	10	0.78	1.49	0.65	0.75	39	111

fermentation, a decrease in pH of 0.3–0.5 pH units was observed for each of the initial cultivation pHs. As the initial acidity of the media increased, the proportion of lactate also increased, while, in parallel, those of the other three products decreased, and vice versa on the alkaline side. For each initial cultivation pH value, carbon recovery of fermentation products against glucose consumed was approximately 100 % and the molar ratios among the three products other than lactate were retained.

An analysis of the fermentation products of *Alkalibacterium olivapovlriticus* NCIMB 13710^T was also conducted and it was found to be a lactic acid bacterium as well, producing mainly lactic acid without gas formation (Table 2). The ratio of the L(+) isomer to total lactate produced was 41 %. Essentially, the same inverse relationship between the proportion of lactate produced and the proportion of the total amount of the other three products, with the molar ratio among them retained, that was exhibited by the new isolates was also observed for *Alkalibacterium olivapovlriticus* NCIMB 13710^T (Table 2).

Lactic acid fermentation accompanied by the production of these three products has been reported previously in *Streptococcus liquefaciens* (Gunsalus & Niven, 1942), *Streptococcus faecalis* (Lindmark *et al.*, 1969), *Streptococcus mutans* and *Streptococcus sanguis* (Carlsson & Griffith, 1974), *Streptococcus lactis* (Thomas *et al.*, 1979), *Lactobacillus bulgaricus* (Rhee & Pack, 1980), *Carnobacterium funditum* and *Carnobacterium alterfunditum* (Franzmann *et al.*, 1991) and *Lactosphaera pasteurii* (Janssen *et al.*, 1995). In *Streptococcus lactis* (now *Lactococcus lactis* subsp. *lactis*) glucose-limited cultivation caused the proportions of formate, acetate and ethanol to increase (Thomas *et al.*, 1979). In *Lactobacillus bulgaricus* (now *Lactobacillus delbrueckii* subsp. *bulgaricus*), fermentation media with an alkaline pH resulted in an increase in the relative amounts of these three products (Rhee & Pack, 1980). The conversion of pyruvate at the end of the Embden–Meyerhof pathway can follow one of two pathways: the formation of lactate mediated by lactate dehydrogenase, or the formation of formate, acetate and ethanol mediated by pyruvate

formate-lyase (Axelsson, 1993). In *Streptococcus mutans*, lactate dehydrogenase is activated by fructose 1,6-diphosphate (Carlsson & Griffith, 1974; Yamada & Carlsson, 1975) and pyruvate formate-lyase is inhibited by triose-phosphate (Takahashi *et al.*, 1982). The decrease in the proportion of lactate produced under glucose-limited conditions was explained by the limited production of these intermediates which caused the reduced activity of lactate dehydrogenase and the retained activity of pyruvate formate-lyase. Although not examined enzymically, the isolates and *Alkalibacterium olivapovlriticus* are assumed to have these two pyruvate pathways as well, and the pathway used would be affected by the pH value of the fermentation medium.

Growth behaviour for NaCl, pH and temperature

The optimum NaCl concentration for growth was between 2.0 % (0.34 M) and 3.75 % (0.64 M) (3.0–5.0 % for strain M13-2^T). For strain M13-2^T, the maximum specific growth rates, μ_{\max} (h⁻¹), were 0.38 in 0 %, 0.36 in 0.5 %, 0.40 in 1.0 %, 0.42 in 1.5, 2.0 and 2.5 %, 0.47 in 3.0 %, 0.51 in 3.75 %, 0.46 in 5.0 % and 0.36 in 7.5 % NaCl. For strain M13-6, as another example, the values were 0.40 in 0 and 0.5 %, 0.42 in 1.0, 1.5 and 2.0 %, 0.40 in 2.5 %, 0.60 in 3.0 %, 0.52 in 3.75 %, 0.44 in 5.0 % and 0.38 in 7.5 % NaCl. Growth occurred in 2.5 % NaCl GYPF broth without NaCl and in those with NaCl concentrations from 17.0 to 20.5 % (2.91–3.51 M). The isolates were slightly halophilic (Kushner, 1978; Kushner & Kamekura, 1988) and highly halotolerant. The only previously reported lactic acid rods which grow optimally at a salinity above 0 % are *Carnobacterium funditum* and *Carnobacterium alterfunditum*: 1.7 and 0.6 %, respectively (Franzmann *et al.*, 1991). These species were isolated from the water of Ace Lake in Antarctica, which has a salinity of about 0.6 (bottom) to 4.3 % (surface) (Burton, 1980) and has been suggested to be of seawater origin (Masuda *et al.*, 1988). They grow optimally at low salinity, which suggests that they may have adapted to salt-containing environments.

The isolates are alkaliphilic (Jones *et al.*, 1994). The initial optimum pH for growth was between 8.0 and 9.5. No

growth was observed in media with an initial pH ≤ 5.5 or ≥ 10.5 . For strain M13-2^T, the maximum specific growth rates, μ_{\max} (h⁻¹), were 0.44 at pH 7.0, 0.48 at pH 7.5, 0.53 at pH 8.0, 0.50 at pH 8.5, 0.51 at pH 9.0, 0.50 at pH 9.5 and 0.34 at pH 10.0. However, the *Carnobacterium* species from the Antarctic lake are neutrophilic, growing best at pH 7.0. When the isolates were grown in 2.5 % NaCl GYPF broth, pH 8.5, the final pH could be as low as 4.7–5.2, which was 0.8 to 1.3 pH units lower than the minimum pH required to initiate growth. It is noteworthy that our isolates preferred alkaline to acidic conditions and were unable to produce particularly acidic conditions as almost all terrestrial lactic acid bacteria are able to do as they prefer weakly acidic reactions and suppress other micro-organisms in their niches by making their surroundings acidic. However, the pH values of 4.7–5.2 attained during the growth of the isolates may be sufficiently low to exclude other bacteria in seawater-associated, alkaline environments. The other alkaliphilic lactic acid bacteria reported so far are *Pediococcus urinaeequi*, which was described isolated from horse urine (Mees, 1934) and has an optimum pH of 9.0 or higher (Nakagawa & Kitahara, 1959), and *Alkalibacterium olivapovlenticus*, which was isolated from alkaline edible-olive wash waters (pH 10.8) and has an optimum pH of 9.0–10.4 (Ntougias & Russell, 2001). Both organisms were isolated from terrestrial, alkaline environments. Our organisms were isolated from marine environments and are slightly halophilic and alkaliphilic, growing preferably under the physico-chemical conditions found in seawater. Thus, they are marine-inhabiting organisms and can be called 'marine lactic acid bacteria'.

The optimum temperature for growth of the isolates was 37–40 °C. The maximum specific growth rates, μ_{\max} (h⁻¹), for strain M13-2^T were 0.36 at 25 °C, 0.52 at 30 °C, 0.60 at 37 °C, 0.42 at 40 °C and 0.02 at 42.5 °C. The isolates grew under a wide range of temperatures from -1.8 °C, the freezing point of seawater, to 40–45.0 °C. The optimum and maximum temperatures were markedly higher than those of usual marine bacteria. The isolates grew very well at lower temperatures with respect to the extent of maximum growth, although growth rates were low. For strain M13-2^T, OD₆₆₀ values were 0.78 at -1.8 °C (21 days incubation), 0.70 at 0 °C (21 days), 0.89 at 5 °C (9 days) and 1.14 at 37 °C (15 h, the optimum temperature). For strain M13-5, they were 0.61 at -1.8 °C (21 days), 0.85 at 0 °C (21 days), 0.98 at 5 °C (9 days) and 1.02 at 37 °C (15 h, the optimum temperature).

Chemotaxonomic characteristics

meso-Diaminopimelic acid was not associated with the cell-wall peptidoglycans of the new isolates or *Alkalibacterium olivapovlenticus* NCIMB 13710^T, as demonstrated by TLC. The purified cell wall of strain M13-2^T contained ornithine, glutamic acid and alanine in a molar ratio of 0.8 : 1 : 1.3, and that of *Alkalibacterium olivapovlenticus* NCIMB 13710^T contained aspartic acid, ornithine, glutamic acid and alanine in a molar ratio of 0.53 : 1.10 : 1 : 1.82. From these results, it

was inferred that strain M13-2^T possessed peptidoglycan type A4 β , Orn-D-Glu, and that *Alkalibacterium olivapovlenticus* NCIMB 13710^T possessed peptidoglycan type A4 β , Orn-D-Asp. Our isolates also differed from those for other related genera: the diamino acid at position 3 is lysine in *Dolosigranulum* (type A4 α , Lys-D-Asp) (Aguirre *et al.*, 1993) and *Desemzia* (type A4 α , Lys-D-Glu) (Stackebrandt *et al.*, 1999), and *meso*-diaminopimelic acid in *Carnobacterium* (type A1 γ , direct cross-linkage) (Collins *et al.*, 1987; Franzmann *et al.*, 1991). Among remotely related lactic acid rods, *Lactobacillus fermentum* and *Lactobacillus pontis* possess ornithine at position 3 (Williams & Sadler, 1971; Vogel *et al.*, 1994), but differ from our isolates by having aspartic acid as the cross-bridge amino acid.

The major cellular fatty acids of strain M13-2^T were characterized by straight-chain saturated and monounsaturated even-carbon-numbered types, with the composition 0.4 % C12:0; 5.2 % C14:0; 0.4 % C15:0; 32.0 % C16:0; 3.1 % C16:1; 19.3 % C16:1 Δ 9; 6.9 % C18:0; 30.2 % C18:1 Δ 9 (oleic acid); 1.1 % C18:2; and 1.4 % C20:1.

Respiratory quinones were not present in strain M13-2^T.

DNA base composition and DNA relatedness

The G + C contents of the DNAs of the eight new isolates fell in a narrow range from 34.6 to 36.2 mol%. These isolates formed a single genomic species sharing 74–100 % of DNA relatedness (Table 3), as was manifested by their possession of almost the same phenotypic features as shown above.

16S rRNA gene sequence analysis

Complete sequences of the 16S rRNA gene, 1458–1479 bases in length, were determined for the isolates. The sequences were aligned and compared with the sequences of 21 species of phylogenetically related bacteria and lactic acid bacteria from the public data libraries. The sequences of all eight isolates were identical over 1458 nt, covering the positions from 40 to 1487 (*E. coli* numbering system; Brosius *et al.*, 1978). Comparison of almost complete sequences of the 16S rRNA genes of the new isolates with homologous sequences from other related genera indicated that the isolates exhibited the highest similarity value to the genus *Alkalibacterium* (96.2 % similarity, 1459 nt compared). The next highest similarity value was to strain WN-16 (94.3 %), followed by *Carnobacterium funditum* (93.2 %) and other species of the genus *Carnobacterium* (92.1–92.6 %). Strain WN-16 is an uncharacterized isolate from the alkaline littoral mud/water of Lake Nakuru in the Kenyan-Tanzanian Rift Valley (Duckworth *et al.*, 1996). Sequence similarity values of the isolates to other genera were lower: *Alloiococcus* (91.1 %), *Dolosigranulum* (90.9 %), *Trichococcus* (91.3–92.0 %) and *Desemzia* (91.9 %). A phylogenetic tree showed that the cluster of new isolates constituted an independent line of descent within the lactic acid bacteria group and placed it in a phylogenetic position most closely related to the genus *Alkalibacterium* (Fig. 2).

Table 3. DNA base composition and DNA relatedness among the new isolates and related lactic acid rods

Strain	DNA base composition (mol% G + C)	Relative binding (%) of DNA from:			
		M13-2 ^T	O21	NCIMB 13710 ^T	IFO 15683 ^T
M13-2 ^T	36.2	100	99	3	3
M13-3	35.7	99			
M13-4	35.7	92			
M13-5	34.6	100			
M13-6	35.7	99			4
M13-7	35.8	88			
O1-1	34.7	74	100	4	
O21	34.6	81		3	
<i>A. olivapovliticus</i> NCIMB 13710 ^T	39.7*			100	
<i>C. divergens</i> IFO 15683 ^T	36.4†				100

*Data from Ntougias & Russell (2001).

†Data from Collins *et al.* (1987).

The new isolates, the genera *Alkalibacterium*, *Alloiococcus* and *Dolosigranulum*, and strain WN-16 constituted a phylogenetic cluster with a high bootstrap value (96.7 %). In the phylogenetic tree, the branching point between the lineage of the new isolates and the lineage composed of the genera *Alloiococcus* and *Dolosigranulum* was closer than the branching point between the lineage of the isolates and the species of the genus *Carnobacterium*, despite the higher sequence similarities to *Carnobacterium* species.

16S rRNA secondary structure

The predicted secondary structure of the V6 region of the 16S rRNA of the new isolates was compared with those of other related genera (Ntougias & Russell, 2001) and proved to be different from all of them (Fig. 3). The terminal ring in the V6 region of the isolates consisted of 10 nt, whereas corresponding rings of the genus *Alkalibacterium* and all other related genera were 2 or 3 nt smaller. The pair of nucleotides at positions 462 and 471 are purine and pyrimidine nucleotides in all other related genera, whereas the nucleotides at these positions are both pyrimidines in our isolates and do not make a pair, resulting in the larger ring. The nucleotides of the isolates at positions 478 and 479 and at their complementary positions were the same as those of the genera *Alkalibacterium* and *Desemzia*, but differed from those of the genera *Alloiococcus*, *Dolosigranulum* and *Carnobacterium*. The V6 region of the isolates possessed the sequence UCGGGU at positions 457–462 and the complementary nucleotide sequence at positions 471–476, which was not the case in the genus *Alkalibacterium* or in other related genera. This difference in the nucleotide sequences seems to be at the genus level, as they also differ among the genera *Alkalibacterium*, *Alloiococcus*, *Dolosigranulum*, *Carnobacterium* (identical for 7/7 species), *Desemzia*, *Trichococcus* (two types for four species), *Vagococcus* (identical for 3/4 species) and *Enterococcus* (identical for 18/26 species).

Distinguishing features of the isolates

With regard to phenotypic characteristics, the isolates were similar to the genus *Alkalibacterium*. No remarkable differences were found in cellular morphology, motility, halophilic property (*Alkalibacterium* has an optimum at 3–5 % NaCl) and salt tolerance (*Alkalibacterium* can grow in 15 % NaCl), fermentation characteristics or the presence of oleic acid among their cellular fatty acids (Ntougias & Russell, 2001). But the isolates could be clearly distinguished from the genus *Alkalibacterium* by the peptidoglycan type, the structure of the V6 region of the 16S rRNA, the optimum and the range of pH for growth (*Alkalibacterium* is obligatory alkaliphilic with a minimum growth pH at 8.0) and the minimum growth temperature (–1.8 °C versus 5 °C). The isolates could be distinguished from the closely neighbouring genera and homofermentative lactic acid rods. They were distinguishable from the genus *Alloiococcus* by cellular morphology, motility, catalase reaction, energy metabolism and DNA base composition (*Alloiococcus* are catalase-positive, non-motile cocci and do not ferment glucose, and the G + C content is 44–45 mol%); from the genus *Dolosigranulum* by cellular morphology and peptidoglycan type (*Dolosigranulum* has non-motile ovoid cells and lysine at position 3 of peptidoglycan); from the genus *Desemzia* by peptidoglycan type and cellular fatty acid composition (*Desemzia* has lysine at position 3 of peptidoglycan and *cis*-vaccenic acid in the fatty acid composition); from homofermentative *Lactobacillus* spp., depending on species, by motility, cellular fatty acid composition and DNA base composition; from the genus *Carnobacterium* by peptidoglycan type (peptidoglycan type of *Carnobacterium* is type A1_γ, direct cross-linkage); and from the genus *Paralactobacillus* by DNA base composition (DNA base composition of *Paralactobacillus* is 45–47 mol% G + C). Except for the genus *Alkalibacterium*, and *Carnobacterium funditum* and *Carnobacterium alterfunditum* the isolates can be distinguished from the genera mentioned

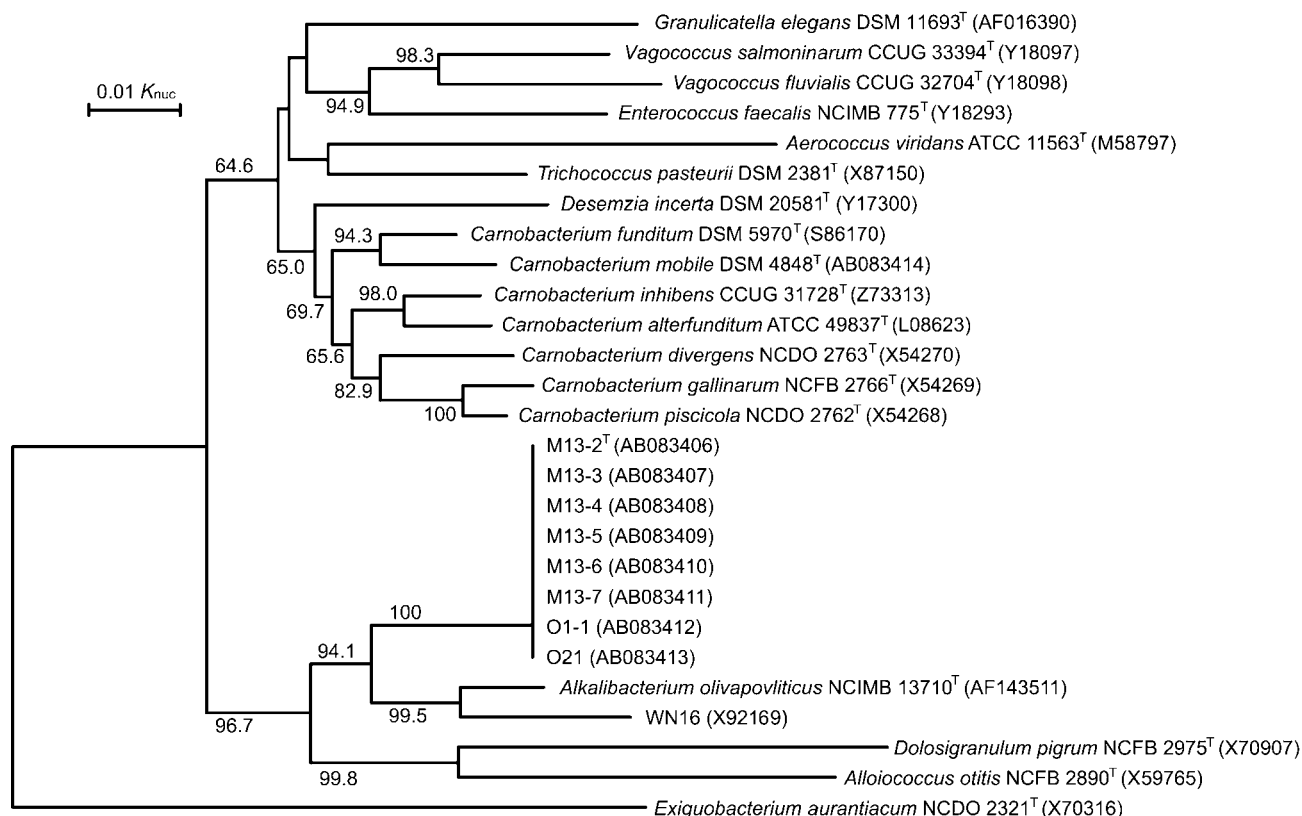


Fig. 2. Phylogenetic relationships between the new isolates and some other related bacteria belonging to the lactic acid group. *Exiguobacterium aurantiacum* NCDO 2321^T was used as an outgroup. The tree, constructed using the neighbour-joining method, is based on a comparison of approximately 1390 nt. Bar, 0.01 K_{nuc} in nucleotide sequences. Bootstrap values, expressed as a percentage of 1000 replications, are given at branching points; only values above 50 % are indicated.

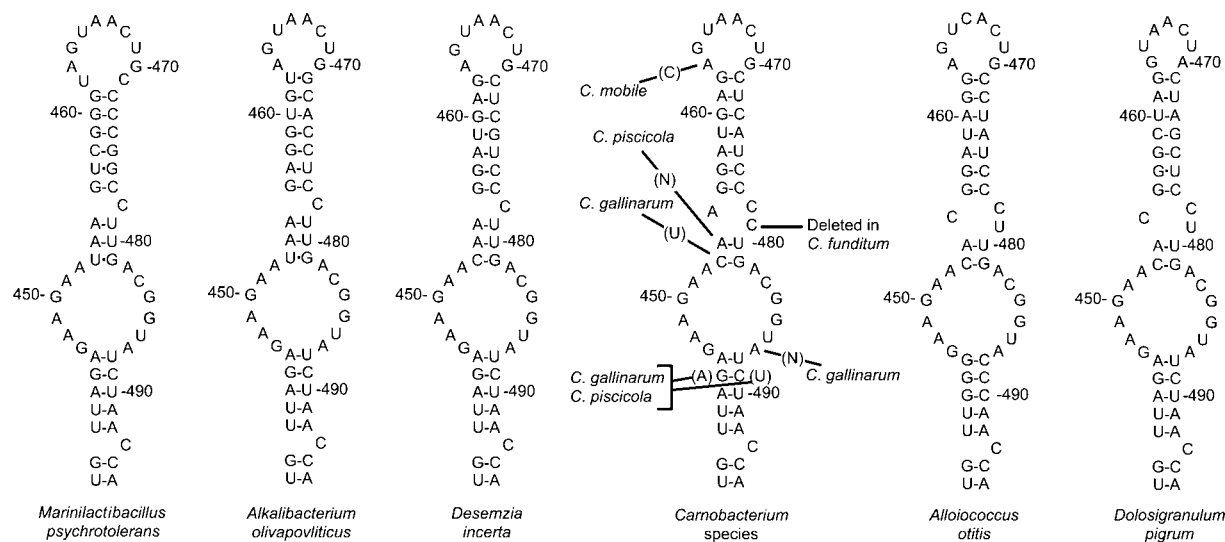


Fig. 3. Nucleotide sequences and secondary structures of the V6 region of the 16S rRNA of the new isolates and closely related bacteria. Numbers correspond to positions in the *E. coli* sequence.

above by all of the characteristics of halophilic and alkaliphilic properties, halotolerance, peptidoglycan type and the structure of the V6 region of the 16S rRNA.

In conclusion, on the basis of phenotypic features, chemotaxonomic characteristics, phylogenetic relationships and the secondary structure and nucleotide sequence of the V6 region of the 16S rRNA, our isolates should be classified as a new genus and species. For the organism we propose the name *Marinilactibacillus psychrotolerans* gen. nov., sp. nov.

Description of *Marinilactibacillus* gen. nov.

Marinilactibacillus (Ma.ri.ni.lac.ti.ba.cil'lus. L. adj. *marinus* marine; L. n. *lactis* milk; L. n. *bacillus* a small rod; N.L. masc. n. *Marinilactibacillus* marine lactic acid rodlet).

Cells are Gram-positive, non-sporulating, straight rods, occurring singly, in pairs or in short chains. Motile with peritrichous flagella. Catalase and oxidase-negative. Negative for nitrate reduction and gelatin liquefaction. Ammonia is weakly produced from L-arginine. Slightly halophilic and highly halotolerant; the optimum NaCl concentration for growth is 2.0–3.75 % (w/v) with a range of 0 to 17.0–20.5 % (w/v) (depending on the strain). Alkaliphilic; the optimum pH for growth is 8.5–9.0 with a range of 6.0–10.0. Growth occurs between –1.8 and 40–45 °C with an optimum temperature of 37–40 °C. L(+) Lactic acid is the major end product from D(+) glucose; trace to small amounts of formate, acetate and ethanol are produced with a molar ratio of approximately 2:1:1, without gas formation. The peptidoglycan is of the A4 β , Orn-D-Glu type. Cellular fatty acids are primarily of the straight-chain saturated and monounsaturated even-carbon-numbered types. The major fatty acids are C16:0, C16:1 Δ 9, C18:0 and C18:1 Δ 9 (oleic acid). Respiratory quinones are absent. The G + C content of the DNA is 34.6–36.2 mol%. The type species of the genus is *Marinilactibacillus psychrotolerans* sp. nov.

Description of *Marinilactibacillus psychrotolerans* sp. nov.

Marinilactibacillus psychrotolerans (psy.chro.to'le.rans. Gr. adj. *psychros* cold; L. part. adj. *tolerans* tolerating; N.L. adj. *psychrotolerans* tolerating cold temperature).

The species has all of the characteristics that define the genus. In addition, it has the characteristics described below. Deep colonies in agar medium are pale yellow, opaque and lenticular with diameters of 2–4 mm. Cells are 0.4–0.5 \times 2.3–4.5 μ m and elongated in older cultures. Grows evenly throughout a column of semi-solid agar medium. Acid is produced from a fairly wide range of carbohydrates, sugar alcohols and related carbon compounds (Table 1). Sodium gluconate is fermented without gas production. The G + C content of the DNA of strain M13-2^T is 36.2 mol%. Isolated from a living sponge, raw Japanese ivory shell and decomposing alga. The type strain has been deposited at the IAM Culture Collection, the Institute of Molecular and Cellular Biosciences, The

University of Tokyo, Tokyo, Japan, the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Kisarazu, Japan, the National Collections of Industrial, Food and Marine Bacteria (NCIMB), Aberdeen, UK and the Nodai Culture Collection Center (NRIC), Tokyo University of Agriculture, Tokyo, Japan, under the accession numbers IAM 14980^T, NBRC 100002^T, NCIMB 13873^T and NRIC 0510^T, respectively.

ACKNOWLEDGEMENTS

We are grateful to Masayuki Suzuki, Mitsui Norin Co. Ltd, for the fatty acid analysis, Toshimori Kadokura, Faculty of Applied Bio-Science, Tokyo University of Agriculture, for the cell wall amino acid analysis, Yuko Itamiya and Shihomi Ishizaki, Faculty of Applied Bio-Science, Tokyo University of Agriculture, for technical assistance, and the Institute for Fermentation, Osaka (IFO), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and the National Collections of Industrial, Food and Marine Bacteria (NCIMB) for supplying the type and reference strains.

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