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Full Paper

Paraliobacillus ryukyuensis gen. nov., sp. nov., a new Gram-positive, slightly halophilic, extremely halotolerant, facultative anaerobe isolated from a decomposing marine alga

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A slightly halophilic, extremely halotolerant, alkaliphilic, and facultatively anaerobic rod bacterium was isolated from a decomposing marine alga collected in Okinawa, Japan. The isolate, designated O15-7^T, was Gram-positive, endospore-forming, catalase-positive, menaquinone-7-possessing bacterium that is motile by peritrichous flagella. The isolate was an inhabitant of marine environments; the optimum NaCl concentration for growth was 0.75–3.0% (w/v) with a range of 0–22.0%, and the optimum pH was 7.0–8.5 with a range of 5.5–9.5. Catalase was produced in aerobic cultivation but not in anaerobic cultivation. Carbohydrate, sugar alcohol or a related carbon compound was required for growth. In aerobic cultivation, the isolate produced pyruvate, acetate and CO_2 from glucose, and in anaerobic cultivation, it produced lactate, formate, acetate and ethanol with a molar ratio of approximately 2 : 1 : 1 for the last three products. No gas was produced anaerobically. Lactate yield per consumed glucose was markedly affected by the pH of the fermentation medium: 51% at pH 6.5 and 8% at pH 9.0. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. Phylogenetically, the isolate occupied an independent lineage within the group composed of the halophilic/halotolerant/alkaliphilic and/or alkalitolerant species in *Bacillus* rRNA group 1 with the highest 16S rRNA gene sequence similarity of 95.2% to the genus *Gracilibacillus.* For this isolate, *Paraliobacillus ryukyuensis* gen. nov., sp. nov. was proposed. The type strain, O15-7^T (G+C=35.6 mol%), has been deposited in the DSMZ, IAM, NBRC, and NRIC (DSM 15140^T=IAM 15001^T=NBRC 10001^T=NRIC 0520^T).

Key Words——alkaliphile; extremely halotolerant; facultative anaerobe; halophile; lactic acid producing bacterium; marine bacteria; *Paraliobacillus ryukyuensis* gen. nov., sp. nov.

Introduction

Isolation and taxonomic and phylogenetic studies performed in this decade have revealed that most Gram-positive, aerobic spore-forming bacteria which have halophilic/halotolerant/alkaliphilic and/or alkalitolerant properties compose a considerably large phylogenetic group within rRNA 1 group of Ash et al. (1991) in the phyletic assemblage of bacteria classically defined as the genus *Bacillus* (Ash et al., 1991; Garabito et al., 1997; Heyndrickx et al., 1999; Lu et al., 2001; Wainø et al., 1999; Zhilina et al., 2001). They have been isolated mostly from saline or hypersaline environments such as saltern, salt lake, soda lake and seawater, and several genera including *Halobacillus*, *Gracilibacillus*, *Oceanobacillus*, *Amphibacillus* and

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Marinococcus have been described (Hao et al., 1984; Lu et al., 2001; Spring et al., 1996; Wainø et al., 1999; Zhilina et al., 2001).

Littoral environments harbor a large number of various kinds of marine plants and animals. Decomposing dead organisms and intestinal contents of living organisms in these environments are rich sources of organic nutrients that support life for heterotrophic microorganisms. These environments provide favorable niches for facultatively anaerobic bacteria, which generally require complex nutrients for growth. Those bacteria are thought to have characteristic properties that reflect the physicochemical nature of seawater [total salt concentration: 3.2–3.8% (w/v), pH 8.2–8.3 (surface)]. These environments are considered to be good sources of those bacteria in reference to the diversity of microorganisms. While Gram-positive, aerobic bacteria have been isolated from coastal seawater and sediments (Bonde, 1981; Hao et al., 1984; Rüger and Richter, 1979; Schlesner et al., 2001; Ventosa et al., 1989), Gram-positive, facultatively anaerobic bacteria from littoral environments have been much less studied taxonomically.

In the isolation and taxonomic studies of Gram-positive bacteria that inhabit marine environments and produce lactic acid, a facultatively anaerobic rod-shaped bacterium belonging to the halophilic/halotolerant/alkaliphilic and/or alkalitolerant group (henceforth referred to as HA group in this paper) was isolated from a decomposing alga. In the present paper, we describe the taxonomic characterization of the isolate, for which the name *Paraliobacillus ryukyuensis* gen. nov., sp. nov. has been proposed.

Materials and Methods

Isolation of strain. Experiments to isolate lactic acid-producing bacteria were conducted using marine materials (living or decomposing algae, fish, sponges, and shellfish) collected from Okinawa Prefecture, a subtropical area of Japan, in September 1998. Strain O15-7^T was obtained from a decomposing alga, taken at a foreshore site near the Oujima islet adjacent to the main island of Okinawa, by enrichment culture using 7% and 18% (for a subsequent second enrichment) NaCl GYPFSK isolation broths. The compositions of these media were as follows (per 1,000 ml): glucose, 10 g; yeast extract, 5 g; Polypepton (Wako Pure Chemical Industries, Osaka, Japan), 5 g; Extract Bonito (Wako Pure Chemical Industries), 5 g; soy sauce, 50 ml; K_2HPO_4, 10 g; NaCl, 60/170 g; sodium thioglycolate, 1 g; salt solution ($MgSO_4 \cdot 7H_2O$, 40 mg; $MnSO_4 \cdot 4H_2O$, 2 mg; and $FeSO_4 \cdot 7H_2O$, 2 mg ml^{-1}), 5 ml; and cycloheximide, 10 mg. The medium was adjusted to pH 7.5 and autoclaved at 110°C for 10 min. A small piece of a decomposing alga was placed in 7% NaCl GYPFSK isolation broth and incubated for 21 days for the first enrichment followed by the second enrichment in 18% NaCl GYPFSK isolation broth for 15 days at 25°C in static culture. Enrichment culture was pour-plated with 12% NaCl GYPFSK isolation agar (2.0% agar) supplemented with 5 g L^{-1} $CaCO_3$.

Media and growth conditions. For the cultivation and taxonomic characterization, 2.5% NaCl GYPF broth (pH 8.5) was used as the basal medium, which was composed of the following (per 1,000 ml): glucose, 10 g; yeast extract, 5 g; Polypepton, 5 g; Extract Bonito, 5 g; K_2HPO_4, 1 g; NaCl, 25 g; sodium thioglycolate, 1 g; and salt solution, 5 ml. The medium was sterilized by filtration. In some experiments, a 2.5% NaCl GYPFK broth was used, which was prepared by increasing the concentration of K_2HPO_4 in a 2.5% NaCl GYPF broth to 1%. The media without sodium thioglycolate were used for aerobic cultivation. Cultivation was performed at 30°C.

Morphological, physiological and biochemical features. Gram staining was conducted using crystal violet-phenol solution (Merck) following the procedure as described elsewhere (Gerhardt et al., 1994). Flagellation was studied for cells grown on 2.5% NaCl GYPFK agar at 20°C for 2 days using the staining method of Nishizawa and Sugawara as described (Alumni Association of The Institute of Medical Sciences, The University of Tokyo, 1988). Spore formation was observed microscopically for cultures grown at 37°C on yeast extract salts agar (pH 7.5) which had the following composition (per 1,000 ml): yeast extract, 5 g; NaCl, 20 g; $MgSO_4$, 5 g; $CaCl_2$, 2 g; K_2HPO_4, 0.1 g; salt solution, 10 ml; and agar, 15 g and Marine agar (Difco). For the catalase test, the evolution of bubbles upon addition of 3% H_2O_2 solution to aerobically or anaerobically grown cells was observed under a stereoscopic microscope. The utilization of carbohydrates and related compounds was observed by incubating in 2.5% NaCl GYPF broth without glucose as the basal medium, to which substrates were added at 1.0% (w/v). The test media were sterilized by filtration except for starch and inulin-added media which were sterilized by autoclav-

ing at 121°C for 15 min. The utilization of ethanol and organic acids other than gluconic acid was examined in aerobic incubation with shaking. Growth was evaluated based on both the amount of acid produced and the turbidity estimated based on absorbance at 660 nm. Other biochemical tests were conducted following the methods described previously (Gerhardt et al., 1994; Okada et al., 1992), with modifications as follows: supplementation with 2.5% NaCl and adjustment of pH to 8.5, or the use of 2.5% NaCl GYPF broth without glucose as the basal medium.

Growth characteristics in relation to NaCl concentration, pH and temperature. The optimum NaCl concentration, pH and temperature and their ranges for bacterial growth were investigated with 2.5% NaCl GYPF broth as the basal medium in static culture. For pH studies, Good's buffers (MES, MOPS, HEPES, EPPS, CHES, and CAPSO) were added at 100–200 mM concentrations to give the same buffering action during a decrease in pH values by 0.5 units. Growth optima were determined based on the maximum specific growth rate, μ_{max} (h^{-1}).

Requirement of glucose for growth. Growth in the presence and absence of glucose was examined by aerobic cultivation using two broth media: 2.5% NaCl GP broth prepared by replacing yeast extract and Extract Bonito in 2.5% GYPF broth with 10 g of Polypepton (final concentration was 15 g L^{-1}), and 2.5% NaCl GCY broth (pH 8.5) composed of 10 g of glucose, 5 g of Vitamin assay casamino acids (Difco), 0.5 g of yeast extract, 25 g of NaCl, 1 g of K_2HPO_4, 5 ml of salt solution, and distilled water in 1,000 ml volume. Aerobic cultivation was conducted with aeration by shaking in a 100 ml cotton plugged conical flask containing 10 ml of the medium on a reciprocal shaker (180 strokes per min).

Glucose products in relation to oxygen and the initial pH of cultivation medium. Products from glucose were investigated by aerobic and anaerobic cultivation. Aerobic cultivation was conducted as described in the above subsection. AnaeroPack-Kenki (CO_2-generated, Mitsubishi Gas Chemical, Tokyo, Japan) was used for anaerobic cultivation. The effect of the initial pH of the cultivation medium on the composition of products from glucose was studied with 2.5% GYPF broth, which was heavily buffered by adding 100 mM Good's buffers: MES, pH 6.5; HEPES, pH 7.0; and CHES, pHs 8.0 and 9.0, in anaerobic cultivation using AnaeroPack-Keep (not CO_2-generated). End products from glucose were analyzed by HPLC.

Chemotaxonomic characteristics and DNA base composition. For the analysis of isoprenoid quinones, cells grown aerobically or anaerobically were freeze-dried and subjected to extraction of the lipid fraction as described earlier (Yamada, 1987; Yamada and Kuraishi, 1982). Molecular species was determined by HPLC (Yamada, 1987). The presence of *meso*-diaminopimelic acid in cell-wall peptidoglycan was determined by TLC as described elsewhere (Hasegawa et al., 1983; Okada et al., 1992). DNA was extracted and purified as previously described (Marmur, 1961; Saito and Miura, 1963). DNA base composition was determined by reverse-phase HPLC (Tamaoka and Komagata, 1984). The isomeric form of lactate produced was determined enzymatically using D- and L-lactic acid dehydrogenases (R-Biopharm, Darmstadt, Germany).

Phylogenetic analysis based on 16S rRNA gene sequence. The 16S rRNA-coding gene fragments of the DNA were amplified by PCR using two primers, 20F (positions 10–26, sense) and 1540R (pos. 1541–1521, antisense) (*E. coli* numbering system [Brosius et al., 1978]), as described elsewhere (Yanagi and Yamasato, 1993). The amplified 16S rRNA gene was sequenced directly using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and an ABI PRISM model 310 genetic analyzer (Perkin-Elmer) with the following five primers: 20F, 1540R, 350F (pos. 341–358, sense), 800F (pos. 803–819, sense), and 900R (pos. 898–879, antisense). The sequences obtained were aligned using the CLUSTAL X program (version 1.8) (Thompson et al., 1997). Percentage similarities among the new isolate and related organisms were calculated for the sequences of 1,356 to 1,503 bases depending on the number of bases available for comparison for each organism. Phylogenetic distances (K_{nuc}) were calculated by the method of Kimura (1980). A phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987). In the phylogenetic analysis, hypervariable regions at positions 66–103 (V1 region) and below 1432 (including V5 region) were omitted. The robustness of individual phylogenetic clusters was estimated by bootstrapping with 1,000 replications (Felsenstein, 1985). The nucleotide sequence of the isolate has been deposited in the DNA Data Bank of Japan (DDBJ). Accession numbers of the isolate deposited and the reference strains from public databases are shown in Fig. 1.

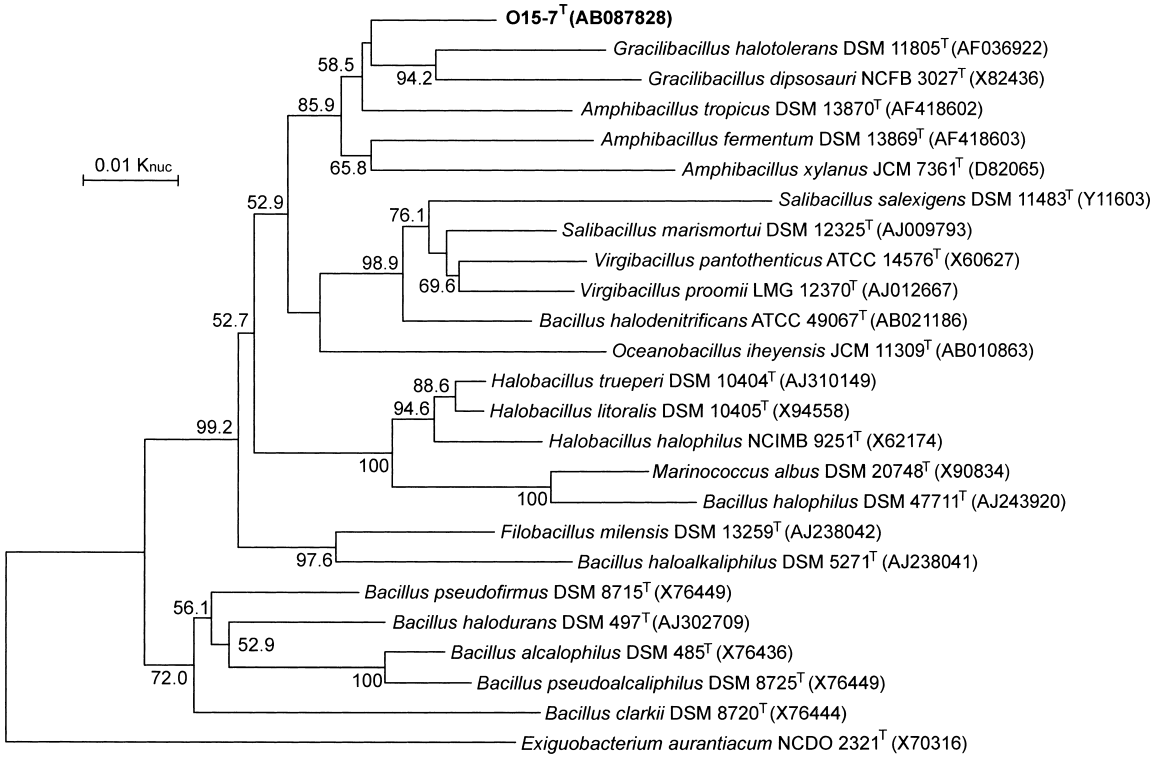

Fig. 1. Phylogenetic relationships between strain O15-7^T and other related bacteria.

Exiguobacterium aurantiacum NCDO 2321^T was used as an outgroup. The tree, constructed using the neighbor-joining method, is based on a comparison of approximately 1,260 nucleotides. Bar, 0.01 K_{nuc} in nucleotide sequences. Bootstrap values, expressed as percentage of 1,000 replications, are given at branching points; only percentages above 50% are indicated.

Results and Discussion

General taxonomic features

Cells of strain O15-7^T were Gram-positive rods that were motile by means of peritrichous flagella (Fig. 2). Spores were formed on yeast extract salts agar (Fig. 3), Marine agar and, with less frequency, yeast extract salts agar in which yeast extract was replaced with Polypepton, yeast extract and Extract Bonito each of which was at 0.17%; Polypepton; or Extract Bonito. Catalase was produced in aerobic cultivation but not in anaerobic cultivation. Pseudocatalase was not produced. The optimum temperature for growth was 37–40°C, with a range of 10–47.5°C. The isolate utilized a wide range of carbohydrates and related compounds tested: pentoses, hexoses, di-, tri- and polysaccharides, sugar alcohols, and their derivatives. Ethanol, formate, lactate, pyruvate, and organic acids involved in the TCA cycle were not utilized in static culture nor in shaking culture. Cultural, morphological, and biochemical features other than those described above and the profile of the utilization of carbohydrates, related compounds, and organic acids are described in the next subsection and the subsection called Description of *Paraliobacillus ryukyuensis* sp. nov.

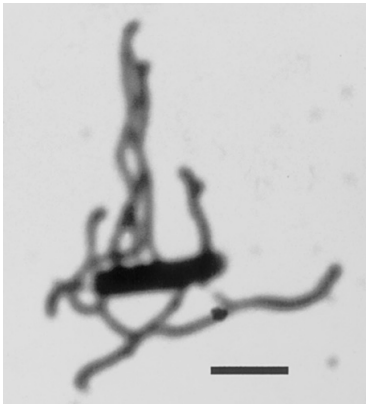

Fig. 2. Photomicrograph of cells and peritrichous flagella of strain O15-7^T grown anaerobically at 20°C for 2 days on 2.5% NaCl GYPFK agar.

Bar, 2 μm.

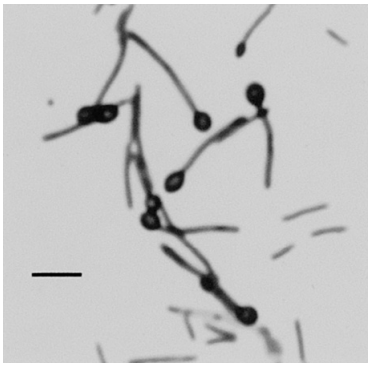

Fig. 3. Photomicrograph of spores and sporangia of strain O15-7^T grown at 37°C for 3 days on yeast extract salts agar. Bar, 5 μm.

Growth characteristics in relation to NaCl concentration and pH

The optimum NaCl concentration for growth of strain O15-7^T was between 0.75% (w/v) (0.13 M) and 3.0% (0.51 M) with specific growth rates, μ_{max} (h^{-1}), of 0.36 to 0.38. These values for other salinities were 0.22 in 0–0.5%, 0.31 in 4.0%, 0.28 in 5%, and 0.20 in 7.0% NaCl. Growth was observed in GYPF broths with 0% and 22.0% (3.8 M) NaCl. The optimum pH for the isolate was between 7.0 and 8.5. The specific growth rates were 0.18 at pH 5.5, 0.36 at pH 6.0, 0.38 at pH 6.5, 0.40 at pH 7.0 to 8.5, and 0.20 at pH 9.0. No growth was observed in the media with an initial pH $\leq$5.0 or $\geq$10.0. When using 2.5% NaCl GYPF broth, the final pH was 4.5 in both aerobic and anaerobic cultivations, which was 1.0 unit lower than the minimum pH for growth initiation (pH 5.5).

Strain O15-7^T is slightly halophilic, as it optimally grows in approximately 0.2–0.5 M NaCl (Kushner, 1978; Kushner and Kamekura, 1988). Jones et al. (1994) defined alkaliphiles as follows: organisms that grow optimally at a pH greater than 8. The isolate can be characterized as 'slightly' alkaliphilic, as it exhibits an optimum growth at neutral to alkaline pH (7.0–8.5), grows well at slightly acidic pH (6.0–6.5) and is able to grow at pH 9.5. It is highly tolerant to an elevated NaCl concentration, comparable to the extremely halotolerant bacteria in the HA group, which is a physiological feature not found in terrestrial forms from non-extreme environments. Strain O15-7^T can be considered to have adapted well to marine environments which are affected by the physicochemical nature of seawater.

Generation of catalase

Generation of catalase was induced by oxygen; the isolate produced catalase on an agar plate or in aerated broth culture, but no catalase activity was detected for anaerobically grown cells. Production of catalase induced by oxygen or hydrogen peroxide has been reported for facultative anaerobes (Finn and Condon, 1975; Hassan and Fridovich, 1978; Loewen et al., 1985). However, these bacteria produced catalase at low level when not subjected to oxidizing agent, and their catalase induction were involved in stimulation or increase of production. Induction of generation of catalase by oxygen has not so far been observed for facultative anaerobes and is considered to be uniquely characteristic of the isolate.

Requirement of glucose (carbohydrate, sugar alcohol or related carbon compounds) for growth

Under aerobic conditions, no growth was observed in 2.5% NaCl GP or 2.5% NaCl GCY broths from which glucose was excluded. Absorbances at 660 nm of 2.5% NaCl GP and 2.5% NaCl GCY broth cultures at the stationary phase were 0.29 and 0.38, respectively, while those in the absence of glucose were 0.03 and below 0.01. Poor growth was observed in 2.5% NaCl GYC broth without glucose and with 0.5% yeast extract, and in 2.5% NaCl GYPF broth without glucose (both absorbances were about 0.10), which was presumably ascribable to some compounds contained in the yeast extract being available to the isolate. Under anaerobic conditions, no growth was observed in 2.5% NaCl GYPF broth without glucose. As the isolate utilizes various kinds of carbohydrates, sugar alcohols and related carbon compounds (the utilization profile is described in the subsection called *Description of Paraliobacillus ryukyuensis sp. nov.*), the isolate requires carbohydrate, sugar alcohol or related carbon compounds for growth, and is not capable of utilizing amino acids or peptides as energy sources through aerobic metabolism. Among the species which ferment glucose in the HA group, three species of the genus *Amphibacillus* cannot grow on media without glucose (carbohydrate and related compounds) under aerobic conditions (Niimura et al., 1989, 1990; Zhilina et al., 2001).

Products from glucose in relation to oxygen and the initial pH of cultivation medium

In aerobic cultivation, acetate, pyruvate, and a small

Table 1. Products from glucose in aerobic and anaerobic cultivations with strain O15-7^T.

Cultivation	Cultivation time (h)	Absorbance at 660 nm	Glucose consumed (mM)	Products (mM)					Carbon recovery (%)
				Pyruvate	Lactate	Formate	Acetate	Ethanol	
Aerobic	11	0.48	5.66	0.55	0.18	ND	5.05	ND	36
	14	1.09	11.32	5.83	0.59	ND	12.16	ND	64
Anaerobic	12	0.36	4.00	ND	0.97	6.90	3.29	3.27	96
	13	0.48	5.33	ND	1.85	9.45	4.59	4.30	103

Strain O15-7^T was cultivated in GYPF broth, pH 8.0, with aeration and under anaerobic condition.
ND: Not detected.

Table 2. Effect of the initial pH of the medium on the composition of products of glucose fermentation by strain O15-7^T.

Initial pH	End products (mol/mol of glucose)				Lactate yield from consumed glucose (%)	Carbon recovery (%)
	Lactate	Formate	Acetate	Ethanol		
6.5	1.03	0.94	0.41	0.37	51	93
7.0	1.00	0.84	0.43	0.48	50	94
8.0	0.41	1.60	0.67	0.65	21	91
9.0	0.16	1.85	0.88	0.93	8	100

Strain O15-7^T was cultivated in heavily buffered 2.5% NaCl GYPF broths with different initial pH values. Decrease in pH during cultivation was 0.5 unit or less.

amount of lactate were produced with a carbon recovery of 36 to 64% depending on cultivation time (Table 1). Formate and ethanol were not detected in cultured broth. CO_2 was produced but the total composition of gaseous products was not analyzed. The carbon recovery at the logarithmic phase (11-h culture) increased about twofold that at the stationary phase (14-h culture), accompanied with much increased accumulation of pyruvate and acetate in the medium.

In anaerobic cultivation, strain O15-7^T produced lactate, formate, acetate, and ethanol from glucose without gas production, with a well-balanced carbon recovery (96–103%) (Table 1). The molar ratio for the latter three products was approximately 2 : 1 : 1. The amount of lactate produced relative to the total amount of the other three products was markedly affected by the pH during cultivation. As the initial pH of the medium was lowered, the relative amount of lactate increased, while the relative total amount of the other three products decreased, and vice versa on the alkaline side (Table 2). For each initial pH, the molar ratio for the three products relative to the lactate produced was substantially retained. *Exiguobacterium aurantiacum*, a facultative anaerobe lying in the phylogenetic radiation of 'classical' *Bacillus*, exhibits the same behavior in glucose fermentation with respect to the change of pH of the cultivation medium (Collins et al., 1983; Gee et al., 1980). Among the HA group, the three species of the genus *Amphibacillus* are similar to the isolate in terms of the metabolism of glucose under anaerobic conditions (Niimura et al., 1989, 1990; Zhilina et al., 2001). *A. xylanus* produces formate, acetate, and ethanol from glucose catalyzed by pyruvate formate-lyase, but not lactate as it lacks lactate dehydrogenase (Niimura et al., 1989). *A. fermentum* and *A. tropicus* also produce formate, acetate and ethanol but not lactate, with carbon recoveries of 92–95% (Zhilina et al., 2001). The effect of different pH of the cultivation medium on the product composition was not studied in these three species.

Alteration of product composition in glucose fermentation, i.e., a decreased lactate production and an increased production of the other three products at an alkaline pH and at limited concentration of glucose in the medium have been reported for homofermentative lactic acid bacteria (Carlson and Griffith, 1974; Gunsu-

lus and Niven, 1942; Rhee and Pack, 1980). Pyruvate is reduced to lactate and to formate, acetate, and ethanol at a molar ratio of 2 : 1 : 1 by lactate dehydrogenase and pyruvate formate-lyase, respectively. The product balance depends on the relative activities of the two enzymes involved (Carlson and Griffith, 1974). The metabolism of glucose of strain O15-7^T under anaerobic condition can be considered to be essentially the same as the altered homolactic fermentation observed in lactic acid bacteria.

Chemotaxonomic characteristics and DNA base composition

meso-Diaminopimelic acid was associated with the cell-wall peptidoglycan. Both of the aerobically and anaerobically grown cells of the isolate possessed menaquinone-7, the amount of which was much larger in aerobic cultivation. The G+C content of DNA was 35.6 mol%. The ratio of the L(+) isomer to the total amount of lactate produced was 52% in 2.5% NaCl GYPFK broth.

16S rRNA gene sequence analysis

The complete sequence of 1,500 bases of the 16S rRNA gene from positions 32 to 1510 (*E. coli* numbering system) was determined. The sequence was aligned and compared with the sequences of 23 species of related bacteria from public databases. Comparison of the homologous sequences and the phylogenetic analysis indicated that strain O15-7^T had the highest 16S rRNA gene sequence similarity (95.1–95.2%) to the genus *Gracilibacillus* and occupied an independent lineage within the HA group in *Bacillus* rRNA group 1 (Fig. 1).

Distinguishing features of strain O15-7^T

Strain O15-7^T, isolated from a decomposing marine alga, is a marine-inhabiting bacterium having slightly halophilic, extremely halotolerant and alkaliphilic properties. These features differentiate it from terrestrial forms from non-extreme environments. Phylogenetically, the isolate occupies a position within the HA group in rRNA group 1 of the phyletic group classically defined as the genus *Bacillus.* This large group is composed of the genera: halotolerant and alkalitolerant *Gracilibacillus*; halophilic, halotolerant and coccal *Marinococcus*; halophilic, halotolerant and alkalitolerant *Oceanobacillus*, *Salibacillus* (Wainø et al., 1999), *Halobacillus* and *Filobacillus*; halophilic and facultatively anaerobic *Virgibacillus*; and the halophilic/halotolerant and/or alkalitolerant species assigned to the genus *Bacillus* (Denariaz et al., 1989; Fritze, 1996; Ventosa et al., 1989) (Fig. 1). *A. xylanus*, a member of this group, was isolated from alkaline compost of manure with grass and rice straw and is neither halophilic nor halotolerant but obligately alkaliphilic (Niimura et al., 1989, 1990). Recently, two halophilic, halotolerant and alkaliphilic/alkalitolerant species have been assigned to the genus *Amphibacillus.*

Strain O15-7^T occupies a phylogenetic position with *Gracilibacillus halotolerans* as the closest neighbor. It differs from all of the members of this group in terms of anaerobic energy metabolism except for *Virgibacillus* and *Amphibacillus*, which ferment glucose, and in terms of glucose (carbohydrate and related compounds) requirement in aerobic conditions except for *Amphibacillus.* In terms of products in glucose fermentation, the isolate is distinguished from *Amphibacillus* which produces formate, acetate and ethanol but not lactate. In addition, the lack of catalase and menaquinone in *A. xylanus* are distinct features that differentiate it from the isolate. The isolate is distinguished from all the members of the HA group by its combination of morphological, physiological, biochemical, and chemotaxonomic features (Table 3).

On the basis of the phenotypic features and phylogenetic independence described above, strain O15-7^T should be classified as a new genus and a new species, for which we propose the name *Paraliobacillus ryukyuensis* gen. nov., sp. nov.

Description of Paraliobacillus gen. nov.

Paraliobacillus (Pa.ra.lio.ba.cil'lus. Gr. adj. *paralios* littoral; L. n. *bacillus* rod; M.L. masc. n. *Paraliobacillus* rod inhabiting littoral [marine] environment).

Cells are Gram-positive, endospore-forming rods that are motile by peritrichous flagella. Endospores are spherical to ellipsoidal and terminal. Sporangia is definitely swollen. Facultatively anaerobic, catalase-positive when cultivated aerobically, pseudocatalase-negative, and requires carbohydrate, sugar alcohol, or related compounds for growth. Glucose is aerobically metabolized to produce acetate and pyruvate as main organic acids. In anaerobic cultivation, lactate, formate, acetate, and ethanol are the end products from glucose, with a molar ratio of approximately 2 : 1 : 1 for the last three products, without gas production. Slightly halophilic and extremely halotolerant. Slightly alka-

liphilic. Contains *meso*-diaminopimelic acid in cell-wall peptidoglycan. The menaquinone type is menaquinone-7. Occupies an independent lineage within the halophilic/halotolerant/alkaliphilic and/or alkalitolerant group in rRNA group 1 of the phyletic group classically defined as the genus *Bacillus*, with a 16S rRNA gene sequence similarity of 95.2% to the genus *Gracilibacillus.* The G+C content of DNA is 35.6 mol%. The type species of the genus is *Paraliobacillus ryukyuensis* sp. nov.

Description of Paraliobacillus ryukyuensis sp. nov.

Paraliobacillus ryukyuensis (ryu.kyu.en'sis. M.L. adj. *ryukyuensis* from the Ryukyu Islands, Japan, where the type strain was isolated).

The species has all of the characteristics that define the genus. In addition, it has the characteristics described below. Colonies on 2.5% NaCl GYPF (glucose-yeast extract-peptone-fish extract) agar are round, convex, entire, yellow and transparent, with diameters of 1.2–1.5 mm after 3 days at 30°C. Deep colonies are creamy white, opaque and lenticular, with diameters of 0.5–1.5 mm. Cells are 0.4–0.5×2.3–4.5 μm occurring singly, in pairs or in chains. Spores are formed on agar media at 37°C, measuring 0.9–1.0× 0.9–1.4 μm. The optimum NaCl concentration for growth is 0.75–3.0% (w/v) with a range of 0–22.0% (w/v). The optimum pH for growth is 7.0–8.5 with a range of 5.5–9.5. Growth is observed at 10–47.5°C with an optimum temperature of 37–40°C. Negative for nitrate reduction, gelatin liquefaction and production of ammonia from L-arginine. Starch is hydrolyzed. The following carbohydrates and related compounds are utilized: D-glucose, D-fructose, D-mannose, L-arabinose, D-xylose, D-ribose, maltose, sucrose, lactose, D-cellobiose, D-trehalose, D-raffinose, D-melezitose, D-mannitol, D-sorbitol, adonitol (weak), dulcitol (weak), glycerol, *myo*-inositol, starch, inulin, D-salicine, α-methyl D-glucoside, and sodium gluconate. D-Galactose, melibiose, D-rhamnose, D-arabinose, ethanol, formate, acetate, lactate, pyruvate, succinate, malate, fumarate, oxaloacetate, 2-oxoglutarate, and citrate are not utilized. The G+C content of the DNA of strain O15-7^T is 35.6 mol%. Isolated from a decomposing alga collected at a foreshore in Okinawa, Japan. The type strain is strain O15-7^T which has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany, the IAM Culture Collection (IAM), the Institute of Mole-

Table 3. Characteristics that distinguish strain O15-7^T from the members of the halophilic/halotolerant/alkaliphilic and/or alkalitolerant group in *Bacillus* rRNA group 1.

Characteristics	1	2	3	4	5	6	7	8	9	10
Cell morphology	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Spore formation	+	+	+	+	+*	+	+	+	+	+
Anaerobic growth	+ (F)	−	+ (ANR)	+ (F)	+ (F)	+ (F)	+ (F)	+ (F)	−	−
Catalase	+†	+	+	−	+	+	+	+	+	+
Glucose requirement in aerobic cultivation	+	−	−	+	+	+	−	−	−	−
NaCl (range, %)	0–22	0–20	0–15	3, +; 6, −	0.98–19.7	0.98–20.9	0–10	0–10	10–20	5–25
NaCl (optimum, %)	0.75–3.0	0	1<	ND	10.8	5.4–10.8	4	4	10	10
pH (range)	5.5–9.5	5–10	6.5–10	8–10	7–10.5	8.5–11.5	ND	ND	7–10	6–9
pH (optimum)	7–8.5	7.5	7.5	ND	8.5–9	9.5–9.7	7	7	7.5	7.5
Major isoprenoid quinone	MK-7	MK-7	MK-7	None	ND	ND	MK-7	MK-7	MK-7	MK-7
Peptidoglycan type	m-Dpm	m-Dpm	m-Dpm	m-Dpm	ND	ND	m-Dpm	m-Dpm	m-Dpm	m-Dpm
G+C content (mol%)	35.6	38	39.4	38	41.5	39.2	36.9	36.8	39.5	39.0–42.8
Isolation source	Decomposing marine alga	Surface mud, Great Salt Lake	Nasal salt glands of a desert iguana	Alkaline grass and rice straw compost	Sediment, soda lake	Sediment, soda lake	Soils	Water supply, soils	Solar salterns, hypersaline soils	Water, Dead Sea

Table 3. Continued

Characteristics	11	12	13	14	15	16	17	18	19
Cell morphology	Cocci	Rods	Rods	Cocci	Rods	Rods	Rods	Rods	Rods
Spore formation	+	+	+	−	+	+	NO	+	+
Anaerobic growth	−	−	−	−	−	−	+ (ANR)	−	ND
Catalase	+	+	+	+	+	+	+	+	+
Glucose requirement in aerobic cultivation	−	−	−	−	−	−	−	−	−
NaCl (range, %)	2–15	0.5–25	0.5–30	5–20	0–21	2–23	2–24.8	3–30	0.5–25
NaCl (optimum, %)	ND	10	10	5–15	3	8–14	2.9–7.9	15	ND
pH (range)	7–9	6–9.5	6–9.5	ND	6.5–10	6.5–8.9	5.8–9.6	6–8	7, −; 9.7, +
pH (optimum)	7.8	7.5	7.5	ND	7–9.5	7.3–7.8	7.4	7	ND
Major isoprenoid quinone	MK-7	MK-7	MK-7	MK-7	MK-7	ND	MK-7	MK-7	MK-7
Peptidoglycan type	Orn-D-Asp	Orn-D-Asp	Orn-D-Asp	m-Dpm	ND	Orn-D-Glu	ND	m-Dpm	m-Dpm
G+C content (mol%)	40.8	42	43	44.9	35.8	35	38	51.5	37, 38
Isolation source	Salt marsh soils	Sediment, Great Salt Lake	Sediment, Great Salt Lake	Solar saltern	Mud, Iheya Ridge	Beach sediment	Solar saltern	Rotting wood on the seashore	Alkaline, highly saline mud

Data from Arahal et al. (1999), Claus et al. (1983), Denariaz et al. (1989), Deutch (1994), Fritze (1996), Garabito et al. (1997), Hao et al. (1984), Heyndrickx et al. (1998, 1999), Lawson et al. (1996), Lu et al. (2001), Niimura et al. (1990), Schlesner et al. (2001), Spring et al. (1996), Ventosa et al. (1989), Wainø et al. (1999), Zhilina et al. (2001) and this study.

1, O15-7^T; 2, *Gracilibacillus halophilus*; 3, *G. dipsosauri*; 4, *Anphibacillus xylanus*; 5, *A. fermentum*; 6, *A. tropicus*; 7, *Virgibacillus pantothenticus*; 8, *V. proomii*; 9, *Salibacillus salexigens*; 10, *S. marismortui*; 11, *Halobacillus halophilus*; 12, *H. litoralis*; 13, *H. trueperi*; 14, *Marinococcus albus*; 15, *Oceanobacillus iheyensis*; 16, *Filobacillus milensis*; 17, *Bacillus halodenitrificans*; 18, *B. halophilus*; 19, *B. haloalkaliphilus*.

+, positive; −, negative; NO, not observed; ND, no data; F, fermentation; ANR, anaerobic respiration; m-Dpm, *meso*-diaminopimelic acid; Orn, ornithine; Asp, aspartic acid; Glu, glutamic acid.

* Spore formation was not observed but culture survived heating.

† Produced in aerobic cultivation.

cular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan, the NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Kisarazu, Japan, and the Nodai Culture Collection Center (NRIC), Tokyo University of Agriculture, Tokyo, Japan, under the accession numbers of DSM 15140^T, IAM 15001^T, NBRC 10001^T, and NRIC 0520^T, respectively.

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