Alkalibacterium subtropicum sp. nov., a slightly halophilic and alkaliphilic marine lactic acid bacterium isolated from decaying marine algae

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Two novel strains of marine lactic acid bacteria, isolated from decaying marine algae collected from a subtropical area of Japan, are described. The isolates, designated O24-2^T and O25-2, were Gram-positive, non-sporulating and non-motile. They lacked catalase and quinones. Under anaerobic cultivation conditions, lactate was produced from glucose with the production of formate, acetate and ethanol in a molar ratio of approximately 2 : 1 : 1. Under aerobic cultivation conditions, acetate and lactate were produced from carbohydrates and related compounds. The isolates were slightly halophilic, highly halotolerant and alkaliphilic. They were able to grow in 0–17.0 % (w/v) NaCl, with optimum growth of strains O24-2^T and O25-2 at 1.0–3.0 and 1.0–2.0 % (w/v) NaCl, respectively. Growth of strain O24-2^T was observed at pH 7.5–9.5, with optimum growth at pH 8.0–8.5. Comparative 16S rRNA gene sequence analysis revealed that the isolates occupied a phylogenetic position within the genus *Alkalibacterium*, showing highest similarity (99.6 %) to *Alkalibacterium putridalgalicola* T129-2-1^T. Although sequence similarity was high, the DNA–DNA relatedness value between strain O24-2^T and *A. putridalgalicola* T129-2-1^T was 27 %, indicating that they are members of distinct species. The DNA G + C contents of O24-2^T and O25-2 were 43.7 and 44.4 mol%, respectively, and DNA–DNA relatedness between the isolates was 89 %. The cell-wall peptidoglycan was type A4 β , Orn-D-Asp. The major cellular fatty acid components were C_{14:0}, C_{16:0} and C_{16:1 ω 9c}. Based on phenotypic characteristics and genetic distinctiveness, the isolates were classified as representatives of a novel species within the genus *Alkalibacterium*, for which the name *Alkalibacterium subtropicum* sp. nov. is proposed; the type strain is O24-2^T (=DSM 23664^T=NBRC 107172^T).

The genus *Alkalibacterium* was proposed by Ntougias & Russell (2001) with *Alkalibacterium olivapoviliticus*, isolated from the wash water of edible olives, as the type species. Subsequently, *Alkalibacterium psychrotolerans* (Yumoto *et al.*, 2004), *Alkalibacterium iburiense* (Nakajima *et al.*, 2005) and *Alkalibacterium indicireducens* (Yumoto *et al.*, 2008) were isolated from polygonum indigo fermentation liquor. Recently, four novel species of the genus *Alkalibacterium* isolated from marine organisms and salted foods were proposed: *Alkalibacterium thalassium*, *Alkalibacterium pelagium*, *Alkalibacterium putridalgalicola* and *Alkalibacterium kapii* (Ishikawa *et al.*, 2009). All species of the genus *Alkalibacterium* possess halophilic and alkaliphilic

properties and produce lactic acid as the main product of glucose fermentation.

Several species of slightly halophilic and alkaliphilic lactic acid bacteria have been isolated from decaying marine organisms. For these bacteria, members of the genera *Marinilactibacillus* (Ishikawa *et al.*, 2003) and *Halolactibacillus* (Ishikawa *et al.*, 2005), and four species of the genus *Alkalibacterium* (Ishikawa *et al.*, 2009), the term ‘marine lactic acid bacteria’ has been proposed on the basis of their marine habitat and preferable growth under the physicochemical conditions found in seawater [total salt concentration, 3.2–3.8 % (w/v); pH 8.2–8.3 (surface)]. During the course of further isolation experiments of lactic acid bacteria from marine environments, novel slightly halophilic and alkaliphilic lactic acid bacteria were isolated from decaying marine algae collected in a subtropical area of Japan. Here, taxonomic characterization of the isolates is described.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains O24-2^T and O25-2 are AB555562 and AB555563, respectively.

Two supplementary figures and three supplementary tables are available with the online version of this paper.

The lactic acid bacteria were isolated from decaying algae that were collected from a foreshore site near the Oujima Islet (26° 07' N 127° 40' E) in Okinawa Prefecture, a subtropical area of Japan, in September 1998.

The lactic acid bacteria isolates were obtained by successive enrichment and subsequent pour-plating following previously described procedures (Ishikawa *et al.*, 2003). For isolation from samples, a 7 % NaCl glucose–yeast extract–peptone–fish extract (GYPF) isolation broth was used for enrichment culture and a 7 % NaCl GYPF isolation agar (1.3 % agar) was used for pour-plating. The compositions of these media were as described previously (Ishikawa *et al.*, 2003), except for pH. The final pH was adjusted to 9.0 by supplementing the media with 6.1 g Na₂CO₃ l⁻¹ and 8.9 g NaHCO₃ l⁻¹. The first enrichment culture was incubated anaerobically at 30 °C for 3 days. For the second enrichment culture, broth medium was inoculated from the first enrichment culture, the pH of which had decreased to <7.0, and incubated anaerobically at 30 °C for 2 days.

Two strains of lactic acid bacteria were isolated from different kinds of marine algae: strain O24-2^T was from *Galaxaura* sp. and strain O25-2 was isolated from *Ectocarpus* sp. *A. olivapovliticus* NCIMB 13710^T, *A. psychrotolerans* JCM 12281^T, *A. iburiense* JCM 12662^T, *A. indicireducens* JCM 14232^T, *A. thalassium* T117-1-2^T, *A. pelagium* T143-1-1^T, *A. putridalgalicola* T129-2-1^T, *A. kapii* T22-1-2^T, *Marinilactibacillus psychrotolerans* M13-2^T and *Marinilactibacillus piezotolerans* JCM 12337^T were used as reference strains. The isolates and reference strains were grown on 2.5 % NaCl GYPF agar (pH 9.0; 1.3 % agar) or in 2.5 % NaCl GYPF broth (pH 9.0) (Ishikawa *et al.*, 2003) at 30 °C unless described otherwise. Anaerobic cultivation was conducted by using AnaeroPack-Keep (not CO₂-generated; Mitsubishi Gas Chemical) as described previously (Ishikawa *et al.*, 2009).

The compositions of media used and methods for characterization of conventional phenotypic features, growth responses to pH, salt concentration in the cultivation medium and temperature, analysis of fermentation products, chemotaxonomic analysis, DNA G+C content, phylogenetic analysis and DNA–DNA relatedness were as described by Ishikawa *et al.* (2002, 2003, 2005, 2009), except where outlined below.

Phylogenetic trees were constructed by using the neighbour-joining method as described previously (Ishikawa *et al.*, 2003, 2005) and the maximum-parsimony method as implemented in the program MEGA version 4.0 (Tamura *et al.*, 2007).

The two isolates exhibited similar phenotypic features. Cells were Gram-positive, rod-shaped and non-motile. Phase-contrast micrographs of cells of the isolates are shown in Supplementary Fig. S1 (available in IJSEM Online). The isolates did not produce spores or catalase. Cultural characteristics, cellular morphology, sensitivity to

antibiotics and other conventional taxonomic features are given in the species description.

Both of the isolates required carbohydrates, sugar alcohols or related compounds for growth under aerobic as well as anaerobic conditions. While the OD₆₆₀ of cultures grown aerobically in 2.5 % NaCl glucose–Casamino acids–yeast extract broth (Ishikawa *et al.*, 2002) was 0.2 to 0.3, the OD₆₆₀ of cultures grown in the same medium without glucose was below 0.05. Similarly, growth was poor in 2.5 % NaCl GYPF broth in which the glucose concentration was reduced from 1 % to 0.1 % and no growth was observed when glucose was omitted from the broth.

The isolates were aerotolerant and slightly sensitive to air. When semi-solid 2.5 % NaCl GYPF medium (0.15 % agar) in a test tube was inoculated with a small amount of broth culture, mixed and incubated statically, the colonies that developed were uniform in both density and size throughout the medium column (about 6 cm in height). When cultivated on an agar medium plate exposed to the atmosphere, growth was less vigorous compared with that on an agar plate incubated anaerobically.

Nearly complete 16S rRNA gene sequences of the isolates were compared with those of lactic acid bacteria and other related bacteria from public databases. The sequences of the two isolates were identical and exhibited highest similarity to the type strain of *A. putridalgalicola* (99.6 %) (Table 1). The next highest similarity values were to the type strains of *A. kapii* (98.0 %), *A. olivapovliticus* (97.9 %), *A. thalassium* (97.8 %), *A. indicireducens* (97.6 %), *A. pelagium* (97.6 %), *A. psychrotolerans* (96.7 %), *A. iburiense* (96.0 %), *M. piezotolerans* (96.0 %) and *M. psychrotolerans* (95.8 %) (Table 1). The phylogenetic tree constructed by using the neighbour-joining method showed that the isolates belonged to a cluster with *A. putridalgalicola* within the radiation of described species of the genus *Alkalibacterium* (Fig. 1). A similar relationship between the isolates and the species of *Alkalibacterium* and *Marinilactibacillus* was also obtained by using the maximum-parsimony algorithm (Supplementary Fig. S2, available in IJSEM Online).

The DNA G+C contents of strain O24-2^T and O25-2 were 43.7 and 44.4 mol%, respectively. DNA–DNA hybridization data showed that these two isolates constituted a single genomic species, sharing 89 % DNA–DNA relatedness. No significant DNA–DNA relatedness (27 %) was obtained between strain O24-2^T and *A. putridalgalicola* T129-2-1^T, which was the closest phylogenetic relative to the isolates (Table 1). The DNA–DNA relatedness values of strain O24-2^T to strains of other species of the genera *Alkalibacterium* and *Marinilactibacillus* were 5–12 %, which are low enough to indicate that they belong to distinct species (Table 1).

The fermentation profiles of carbohydrates, sugar alcohols and related carbon compounds of the isolates are given in the species description. The isolates utilized a fairly wide range of substrates. Both isolates utilized starch and gluconate without gas production.

Table 1. Sequence similarities and DNA–DNA relatedness of the novel isolates and related taxa

Strain	16S rRNA gene sequence similarity value (%) with strain O24-2 ^T	Relative binding (%) with DNA from strain O24-2 ^T *	
		Mean value (%)	Standard deviation
O25-2	100	89	3.2
<i>A. putridalgalicola</i> T129-2-1 ^T	99.6	27	5.3
<i>A. kapii</i> T22-1-2 ^T	98.0	12	8.3
<i>A. olivapovlenticus</i> NCIMB 13710 ^T	97.9	7	1.0
<i>A. thalassium</i> T117-1-2 ^T	97.8	8	5.8
<i>A. indicireducens</i> JCM 14232 ^T	97.6	9	2.8
<i>A. pelagium</i> T143-1-1 ^T	97.6	9	7.6
<i>A. psychrotolerans</i> JCM 12281 ^T	96.7	6	2.5
<i>A. iburiense</i> JCM 12662 ^T	96.0	10	2.0
<i>M. piezotolerans</i> JCM 12337 ^T	96.0	5	1.3
<i>M. psychrotolerans</i> M13-2 ^T	95.8	7	1.2

*Values obtained from three repetitions, except for *A. putridalgalicola* T129-2-1^T, in which four repetitions were carried out.

The isolates were slightly halophilic (Kushner, 1978; Kushner & Kamekura, 1988) and halotolerant. The optimum NaCl concentration for growth of strain O24-2^T was between 1.0 and 3.0 % (1.0–2.0 % for strain O25-2) when GYPF broth (pH 8.5) was used and cells were cultivated statically at 30 °C. The maximum specific growth rates ($\mu_{\max}$) of strain O24-2^T were 0.29, 0.30, 0.38, 0.42, 0.41, 0.41, 0.26, 0.26, 0.22, 0.14 and 0.06 h⁻¹ in 0.25, 0.5, 0.75, 1.0,

2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 % NaCl, respectively. The isolates were able to grow in 0 % (weak) to 17.0 % NaCl. No growth was observed at 18.0 % NaCl.

The isolates were alkaliphiles according to Jones *et al.* (1994): alkaliphiles are organisms that grow optimally at pH values greater than 8. The optimum pH for growth was between 8.0 and 8.5 when 2.5 % NaCl GYPF broth was

Fig. 1. Phylogenetic relationships between the novel isolates and related bacteria. The tree, constructed by the neighbour-joining method, is based on comparison of approximately 1400 nt. Bootstrap values, expressed as percentages of 1000 replications, are given at branch points; only values above 50 % are shown. Bar, 0.01 K_{nuc} .

used and cells were cultivated statically at 30 °C. The $\mu_{\max}$ values of strain O24-2^T were 0.18, 0.60, 0.54, 0.37 and 0.36 h⁻¹ at pH 7.5, 8.0, 8.5, 9.0 and 9.5, respectively. No growth was observed in media with initial pH ≤ 7.0 for strain O24-2^T (≤ 7.5 for strain O25-2) or ≥ 10.0 .

Commonly occurring lactic acid bacteria are comparatively acid tolerant (their broth cultures attain a final pH of <3.0) and grow optimally at neutral to slightly acid pH, such as 6.0. The isolates and members of the genus *Alkalibacterium* are not acid tolerant or neutralophilic, but they are alkaliphilic, as are *M. psychrotolerans* and *Halolactibacillus* species, which is a characteristic that is not present in common terrestrial lactic acid bacteria.

Among the genus *Alkalibacterium*, *A. thalassium*, *A. pelagium*, *A. putridalgalicola* and *A. kapii* are marine inhabitants, isolated from marine organisms or salted foods produced from marine organisms, and possess halophilic and alkaliphilic properties consistent with those of seawater. The isolates reported here can be regarded as additional representatives of the marine lactic acid bacteria (Ishikawa *et al.*, 2003, 2005, 2009) on the basis of habitat, physiological properties and lactic acid fermentation.

The optimum growth temperature of the isolates was 20–30 °C when 2.5 % NaCl GYPF broth (pH 8.5) was used and cells were cultivated statically. The isolates grew at 15–40 °C; no growth was observed at ≤ 10 °C or ≥ 42.5 °C. The $\mu_{\max}$ values of strain O24-2^T were 0.24, 0.28, 0.28, 0.28, 0.18 and 0.16 h⁻¹ at 15, 20, 25, 30, 37 and 40 °C, respectively.

Fermentation products from glucose were analysed for anaerobic cultures grown in 2.5 % NaCl GYPF broth. Lactate, formate, acetate and ethanol were produced without generation of gas; formate, acetate and ethanol were produced in a molar ratio of approximately 2:1:1. The ratios of L(+) isomer to total lactate produced were 51 and 47 % for strains O24-2^T and O25-2, respectively. The effect of the initial pH of the fermentation medium on the yield of lactate produced (mol glucose consumed)⁻¹ was investigated by using media buffered with a series of Good's buffers (Ishikawa *et al.*, 2002) for the isolates (Supplementary Table S1, available in IJSEM Online). To minimize the effect of pH changes that might occur during cultivation, product analysis was performed when cultures were at an early stage of growth (i.e. OD₆₆₀ of approx. 0.2). As the initial acidity increased, the lactate yield relative to glucose consumed by strain O24-2^T increased, as observed in strains of the genera *Alkalibacterium* and *Marinilactibacillus*: 93 % at pH 7.5, 55 % at pH 8.0 and 26 % at pH 9.0. Strain O25-2 produced similar amounts of lactic acid regardless of pH: 45 % at pH 8.0 and 38 % at pH 9.0. At all pH values, carbon recovery from consumed glucose was about 100 % and the molar ratios of formate, acetate and ethanol were generally approximately 2:1:1 (Supplementary Table S2, available in IJSEM Online). Several homofermentative lactic acid bacteria, including members of the genera *Alkalibacterium* and *Marinilactibacillus*, have been reported to produce formate, acetate and ethanol, in addition to lactate, from

glucose in a molar ratio of 2:1:1. This phenomenon can be explained as follows. These homofermentative lactic acid bacteria possess pyruvate-associated enzymes, lactate dehydrogenase and pyruvate-formate lyase (Carlsson & Griffith, 1974; Axelsson, 1993). Relative activities of these enzymes are affected by the pH of the fermentation medium or limited glucose concentration resulting in the generation of varying relative amounts of lactate and the other three products (Gunsalus & Niven, 1942; Carlsson & Griffith, 1974; Yamada & Carlsson, 1975; Rhee & Pack, 1980). As discussed for species of *Marinilactibacillus* (Ishikawa *et al.*, 2003), *Halolactibacillus* (Ishikawa *et al.*, 2005) and *Alkalibacterium* (Ishikawa *et al.*, 2003, 2009), the isolates may have these two pyruvate pathways and, in strain O24-2^T, the relative activities of the enzymes involved would be affected by the pH of the fermentation medium.

The isolates grew aerobically, although they lacked catalase and respiratory quinones. The end products from glucose under aerobic conditions were determined for strain O24-2^T (Supplementary Table S2). Acetate and lactate were produced in molar ratios of 0.81 and 1.07 (per mol glucose consumed), respectively, but formate and ethanol were not generated. Carbon recovery was 81 %. If we assume that equimolar production of acetate and CO₂ occurs, carbon recovery can be calculated as 95 %. Oxidative generation of equimolar amounts of acetate and CO₂ from glucose that is not mediated by the respiratory chain has been reported for several other lactic acid bacteria (Fukui *et al.*, 1988; Sakamoto & Komagata, 1996; Liu *et al.*, 2002). The isolates would have a relevant oxidative pathway of glucose as in the case of species of the genera *Marinilactibacillus* (Ishikawa *et al.*, 2005), *Halolactibacillus* (Ishikawa *et al.*, 2005) and *Alkalibacterium* (Ishikawa *et al.*, 2009).

meso-Diaminopimelic acid was not associated with cell-wall peptidoglycans of the isolates as determined by TLC. Purified cell wall of strain O24-2^T contained aspartic acid, glutamic acid, alanine and ornithine in a molar ratio of 0.44:1.0:1.7:1.0. From this result, it was inferred that strain O24-2^T possessed peptidoglycan type A4 β , Orn-D-Asp, which is the cell-wall type of *A. olivapovliticus* (Ishikawa *et al.*, 2003), *A. thalassium* (Ishikawa *et al.*, 2009), *A. pelagium* (Ishikawa *et al.*, 2009) and *A. putridalgalicola* (Ishikawa *et al.*, 2009).

The major cellular fatty acids of strain O24-2^T were straight-chain saturated and monounsaturated even-carbon-numbered types, as follows: 2.7 % C_{10:0}; 13.8 % C_{14:0}; 23.9 % C_{16:0}; 46.1 % C_{16:1 ω 9}; 0.6 % i-C_{17:1 ω 9}; 1.1 % C_{18:0}; 7.5 % C_{18:1 ω 9}; and 4.3 % unknown fatty acid. The cellular fatty acid compositions of members of the *Alkalibacterium* are generally similar to each other (Supplementary Table S3, available in IJSEM Online). However, the proportion of C_{14:0} in the cellular fatty acid composition of strain O24-2^T is relatively high compared with that found in other members of *Alkalibacterium* except for *A. psychrotolerans* (Supplementary Table S3).

Respiratory quinones were not present in strain O24-2^T.

Table 2. Differential characteristics of the novel isolates and phylogenetically related species

Taxa: 1, *Alkalibacterium subtropicum* sp. nov.; 2, *A. thalassium* (Ishikawa *et al.*, 2009); 3, *A. pelagium* (Ishikawa *et al.*, 2009); 4, *A. putridalgicola* (Ishikawa *et al.*, 2009); 5, *A. kapii* (Ishikawa *et al.*, 2009); 6, *A. olivapovlenticus* (Ntougias & Russell, 2001); 7, *A. psychrotolerans* (Yumoto *et al.*, 2004); 8, *A. iburiense* (Nakajima *et al.*, 2005); 9, *A. indicireducens* (Yumoto *et al.*, 2008); 10, *M. psychrotolerans* (Ishikawa *et al.*, 2003); 11, *M. piezotolerans* (Toffin *et al.*, 2005). +, Positive; –, negative; w, weak; v, variable reaction; R, resistant; S, susceptible; WS, weakly susceptible; ND, no data.

Characteristic	1	2	3	4	5	6	7	8	9	10	11
Motility	–	–	+	+	+	+	+	+	+	+	–
Flagella	–	–	Peritrichous	Peritrichous	(most strains) Peritrichous	Peritrichous*	Peritrichous	Peritrichous	Peritrichous	Peritrichous	–
Colony colour	Creamy white	Pale yellow	Pale yellow	Pale yellow	Creamy yellow	Yellowish white*	Pale white	Pale white	Pale white	Pale yellow	Yellowish white*†
NaCl (%)											
Range	0–17	0–11	0–17	0 to 18–20	0 to 19–21	0–15	0–17	0 to 14–16	0 to 14–15	0 to 17–20.5	0–12.0
Optimum	1.0 to 2.0–3.0	1–2.5	0.5–1.5	2–4	1.5–2.5	3–5‡	2–12	3–13	1–11	2–3.75	1.0–2.0
pH											
Range	7.5–8.0 to 9.5	7.0–11.0	7.0–11.0	6.0–6.5 to 10	6.5–10.0	8.5–10.8	9.0–12.0	9.0–12.0	9.0–12.3	6.0–10.0	5.5–10.0
Optimum	8.0–8.5	9.0	9.0–9.5	8.0–9.0	8.5–9.0	9.0–10.2	9.5–10.5	9.5–10.5	9.5–11.5	8.5–9.0	7.0–8.0
Growth temperature (°C):											
Range	15–40	10–42.5	10–47.5	–1.8 to 40–45	5–10 to 40–42.5	4 to 35–37	5–45	5–45	15–40/18–37	–1.8 to 40–45	4–50
Optimum	20–30	37	37	37–40	25–37	27–32	34	30–37	20–30	37–40	37–40
Fermentation of:											
D-Xylose	+	–	+	+	–	–	+	V	–	+	+
Sucrose	+	+	+	+	+	+	–	V	W	+	+
Lactose	+	W	+	+	–	–	–	+	ND	+ or W	–*
Raffinose	+	W	W	+	–	–	–	–	–	V	–*
D-Salicin	+	+	+	+	–	+	+	+	ND	V	+
Melibiose	+	–	–	–	–	–	–	–	–	V	ND
Inulin	+	+	+	+ or –	–	ND	–	ND	ND	V	ND
Antibiotic sensitivity											
Ampicillin (10 and 25 µg)	S	S	S	S	S	S	S*	S	S	S*	S*
Chloramphenicol (2 µg)	WS	R	R	R	R	S	WS*	R	R	R*	R*
Kanamycin (2 and 10 µg)	S	R	R	R	R	S	R	R	R	R*	R*
Trimethoprim (10 and 25 µg)	S	R	S	S	R	S	S*	R	R	R*	R*
NH ₃ from L-arginine	–	–	–	+	–	W*	W*	W*	ND	W	W*
Peptidoglycan type	A4β, Orn-D-Asp	A4β, Orn-D-Asp	A4β, Orn-D-Asp	A4β, Orn-D-Asp	A4β, Orn-D-Glu	A4β, Orn-D-Asp	A4α, Lys (Orn)-D-Asp	A4α, Lys-D-Asp	A4α, Lys (Orn)-D-Asp	A4β, Orn-D-Glu	ND
Major cellular fatty acids	C _{14:0} , C _{16:0} , C _{16:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{14:0} , C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}	C _{16:0} , C _{16:1ω9c} , C _{18:1ω9c}
DNA G + C content (mol%)	43.7–44.4	41.7	42.2	41.0–43.0	38.4–39.4	39.7 ± 1.0	42.6–43.2	40.6 (39.2*)	47.0–47.8	34.6–36.2	42.0 (36.6*)

*Determined by Ishikawa *et al.* (2009).

†Cultivated anaerobically on 2.5 % NaCl GYPF agar.

‡Values for the type strain.

The isolates were similar to members of the genus *Alkalibacterium* in the following characteristics: cellular morphology, various biochemical features, halophilic/halotolerant and alkaliphilic/alkalitolerant properties, products from glucose under aerobic and anaerobic conditions, and DNA G+C content. Although they shared important taxonomic features, the isolates and described species of the genus *Alkalibacterium* can be distinguished from each other by a combination of features, i.e. sugar fermentation patterns, sensitivity to antibiotics, growth responses to NaCl concentration, pH and temperature, the ability to produce ammonia from arginine, peptidoglycan type and DNA G+C content (Table 2). The isolates could be distinguished from described species of *Alkalibacterium* by motility (except for *A. thalassium*) and antibiotic sensitivity (except for *A. olivapovliticus*), and from *A. kapii*, *A. psychrotolerans*, *A. iburiense* and *A. indicireducens* by peptidoglycan type (Table 2). Above all, the isolates could be distinguished from *A. putridalgalicola*, which is the phylogenetically most closely related species, by motility, colony colour, temperature range for growth, sugar fermentation pattern, sensitivity to antibiotics, production of ammonia from L-arginine and major cellular fatty acids (Table 2).

In conclusion, on the basis of the phenotypic and genetic features that we have described, the isolates should be classified as representatives of a novel species of the genus *Alkalibacterium* for which the name *Alkalibacterium subtropicum* sp. nov. is proposed.

Description of *Alkalibacterium subtropicum* sp. nov.

Alkalibacterium subtropicum (sub.tro'pi.cum. L. pref. *sub* under, below, slightly; L. neut. adj. *tropicum* tropical; N.L. neut. adj. *subtropicum* subtropical, referring to the subtropical region where strains were isolated).

Cells are Gram-positive, non-sporulating, non-motile, straight rods measuring $0.5\text{--}0.9 \times 3.0\text{--}20.0\text{ }\mu\text{m}$, occurring singly, in pairs or in short chains and elongated in older cultures (Supplementary Fig. S1). Deep colonies in 2.5 % NaCl GYPF agar medium are creamy white, opaque and lenticular, with diameters of 1.0–2.0 mm after 3 days at 30 °C. Surface colonies are round, convex, entire, creamy white and transparent, with diameters of 0.5–1.0 mm after 3 days at 30 °C. Aerotolerant. Grows evenly throughout a column of semi-solid agar medium. Catalase- and oxidase-negative. Quinones are not detected. Negative for nitrate reduction, gelatin liquefaction and production of ammonia from L-arginine. Slightly halophilic; optimum NaCl concentration for growth is 1.0–3.0 % (type strain); grows in 0–17.0 % (w/v) NaCl. Alkaliphilic; the optimum pH for growth is 8.0–8.5; grows at pH 7.5–9.5 (type strain). Growth occurs between 15 and 40 °C, with optimum growth at 20–30 °C. Lactic acid is the major fermentation product from D-glucose; other products are formate, acetate and ethanol in a molar ratio of approximately 2:1:1, without gas formation. Lactate produced is of DL-type. D-Glucose is aerobically metabolized to acetate and lactate (analysed for

the type strain). The following carbohydrates and related compounds are fermented: D-ribose, D-xylose, D-glucose, D-fructose, D-mannose, cellobiose, D-lactose, maltose, melibiose, sucrose, trehalose, raffinose, D-mannitol, D-sorbitol (weak for O25-2), D-dulcitol (weak), glycerol (weak), D-salicin, sodium gluconate, starch and inulin. D-Arabinose and L-rhamnose are not fermented. Variable reactions are observed for L-arabinose, D-galactose, melezitose, adonitol, myo-inositol and methyl α -D-glucoside. Growth is inhibited by ampicillin (10 μg), chloramphenicol (weakly; 2 μg), kanamycin (2 μg) and trimethoprim (10 μg). The peptidoglycan is A4 β , Orn-D-Asp type. Major cellular fatty acids are C_{14:0}, C_{16:0} and C_{16:1} ω 9c.

The type strain is O24-2^T (=DSM 23664^T=NBRC 107172^T), isolated from a decaying marine alga, *Galaxaura* sp., collected at a foreshore site near the Oujima Islet in Okinawa Prefecture, a subtropical area of Japan. The DNA G+C content of strain O24-2^T is 43.7 mol%.

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