Alkalibacterium iburiense sp. nov., an obligate alkaliphile that reduces an indigo dye

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Three indigo-reducing obligately alkaliphilic strains, M3^T, 41A and 41C, were isolated. The isolates grew at pH 9–12, but not at pH 7–8. They were Gram-positive, facultatively anaerobic, straight rod-shaped strains with peritrichous flagella. The isolates grew in 0–14 % (w/v) NaCl, with optimum growth at 3–13 %. They grew at temperatures between 10 and 45 °C, with optimum growth at around 30–37 °C. They did not hydrolyse starch or gelatin. DL-Lactate was the major end-product from D-glucose. No quinones could be detected. The peptidoglycan type was A4 β , Orn–D–Asp. The major cellular fatty acids were C_{16:0}, C_{16:1}7c and C_{18:1}9c. The DNA G + C content was 42.6–43.2 mol%. Phylogenetic analysis based on 16S rRNA gene sequence data indicated that the isolates belong to the genus *Alkalibacterium*. DNA–DNA hybridization revealed low similarity (less than 16 %) of the isolates with respect to the two closest phylogenetically related strains, *Alkalibacterium olivapovlenticus* and *Alkalibacterium psychrotolerans*. On the basis of phenotypic and chemotaxonomic characteristics, phylogenetic data and DNA–DNA relatedness, the isolates merit classification as a novel species of the genus *Alkalibacterium*, for which the name *Alkalibacterium iburiense* is proposed. The type strain is M3^T (= JCM 12662^T = NCIMB 14024^T).

Alkaliphilic bacteria have been isolated in order to investigate their diversity in terms of environments, taxonomy, physiological adaptation to high pH and the industrial application of their enzymes (Horikoshi, 1991; Duckworth *et al.*, 1996; Takami *et al.*, 1997; Krulwich & Guffanti, 1989; Krulwich *et al.*, 2001; Yumoto, 2002, 2003; Thongaram *et al.*, 2003). Most of the strains used for investigation and industrial utilization belong to the genus *Bacillus*. Such alkaliphilic bacteria have been isolated mostly from soil samples and other environments, e.g. fermentation process and water environments. Alkaliphilic bacteria are distributed not only in soil but also in sea water, fresh water, the intestines of insects, alkaline soda lakes and deep-sea and artificial environments (Nielsen *et al.*, 1995; Duckworth *et al.*, 1996; Takami *et al.*, 1997; Thongaram *et al.*, 2003;

Ntougias & Russell, 2001; Yumoto *et al.*, 2004). In artificial environments, alkaliphilic bacteria belonging to genera other than *Bacillus* also exist.

The traditional method for producing indigo blue dye had been based on fermentation by alkaliphilic bacteria existing in ubiquitous environments in Japan. The harvested indigo leaves are air-dried and then appropriately wetted before being further processed using moisture-controlled bacterial oxidation. The product obtained is further processed by microbial reduction under alkaline conditions (pH values above 10), at which point the original insoluble oxidized form of indigo is converted to a soluble reduced form. The fermentation method for producing indigo dye by using alkaliphilic bacteria had declined by around 1960 because of the difficulty in managing the fermentation process. Then, a chemical reducing reagent was introduced to replace the bacterial reduction. If we can understand the characteristics of these bacteria in relation to fermentation, it might be easier to manage the fermentation process. A return to the traditional method might diminish the use of the chemical reagent. Indigo-reducing bacteria have been isolated by Takahara & Tanabe (1960) and Padden *et al.* (1999) and have been identified as a *Bacillus* species and *Clostridium isatidis*, respectively. In addition to strains M3^T, 41A and

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains M3^T, 41A and 41C are AB188091, AB188092 and AB188093, respectively.

A transmission electron micrograph showing the peritrichous flagellation of strain M3^T is available as a supplementary figure in IJSEM Online, along with a supplementary table showing DNA–DNA relatedness among the strains examined in this study.

41C, we have also isolated *Alkalibacterium psychrotolerans*, which is an indigo-reducing bacterium that has recently been described taxonomically (Yumoto *et al.*, 2004).

In this study, halotolerant, psychrotolerant, obligately alkaliphilic strains that can reduce indigo at high pH values were isolated and characterized. Phenotypic and chemotaxonomic characteristics, phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization showed that the isolates merit classification as a novel species of the genus *Alkalibacterium*.

Fermented polygonum indigo (*Polygonum tinctorium* Lour.) samples were obtained from Date city (42° 42' N, 140° 42' E), Hokkaido, Japan, on 5 July 2002. At the same time, 5 ml of the fermentation liquor was inoculated into 100 ml 0.01 % indigo-containing PYA (peptone/yeast extract/alkaline) broth consisting of 8 g peptone (Kyokuto), 3 g yeast extract (Merck), 1 g K₂HPO₄, 3.5 mg EDTA, 3 mg ZnSO₄·7H₂O, 10 mg FeSO₄·7H₂O, 2 mg MnSO₄·5H₂O, 1 mg CuSO₄·5H₂O, 2 mg Co(NO₃)₂·6H₂O and 1 mg H₃BO₃ in 1 l NaHCO₃/Na₂CO₃ buffer (100 mM in deionized water; pH 10) at 27 °C. After checking for microbial reduction, the culture was transferred to another 100 ml fresh 0.01 % indigo-containing PYA broth. This enrichment process was performed five times using the same medium. During the screening process involving about 40 isolates and our laboratory strains, one alkaliphilic laboratory strain was contaminated with a micro-organism that also tested positive for indigo reduction. This fraction was also processed by using the enrichment process described above. The enrichment culture thus obtained was inoculated onto a reinforced clostridial agar (RCA; Sigma) plate containing 100 mM NaHCO₃/Na₂CO₃ buffer at pH 10 (alkali-RCA) and incubated in an argon exchange jar. Although one strain (M3^T) was isolated independently from contaminated culture in our laboratory, two strains (41A and 41C) were isolated as colonies with different features from the same sample and using the same isolation process. After 48 h cultivation, single colonies were picked and reisolated five times using alkali-RCA plates. The purified colonies obtained were inoculated into 0.01 % indigo-containing PYA broth to check the ability of the isolate to reduce indigo. Cells for chemotaxonomic analysis were harvested in the late-exponential phase after cultivation with reciprocal shaking (130 r.p.m. min⁻¹) at 27 °C in reinforced clostridial broth (RCB; Sigma) containing 100 mM NaHCO₃/Na₂CO₃ buffer at pH 10 (alkali-RCB). Alkali solution for media used in this study was sterilized separately by autoclaving.

For phenotypic characterization, alkali-RCA or alkali-RCB was used as the basal medium. The culture was incubated at 27 °C for 2 weeks and experiments were performed three times to confirm the reproducibility of results. Acid production from carbohydrates was determined by the method of Hugh & Leifson (1953), using thymol blue (0.008 %, w/v) instead of bromothymol blue at pH 10. Growth experiments at pH 7–10 were performed using PYA

medium containing 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7–8), 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9–10) or 100 mM Na₂CO₃/NaOH buffer (pH 11–12.3). Anaerobic growth was tested in PYA broth (pH 10) by substituting air with argon gas. Other physiological and biochemical characteristics were examined according to the methods of Yumoto *et al.* (1998). Antibiotic sensitivity was investigated by placing a paper disc, impregnated with a small amount of antibiotic, on a plate of alkali-RCA medium. For the observation of negatively stained cells by transmission electron microscopy (model H-800 microscope; Hitachi), cells were grown on alkali-RCA; preparation and observation were performed as described previously (Yumoto *et al.*, 2001). The morphological, physiological and biochemical characteristics of the isolate are given in the species description. The isolates grew almost equally well at pH values 9–12, but not at pH 7–8, the optimum pH being 9.5–10.5. They were facultatively anaerobic. Three strains were isolated using alkali-RCA (pH 10). Colonies of strains M3^T, 41A and 41C on an alkali-RCA plate were circular, convex and pale white; the cells of the isolates were Gram-positive, peritrichously flagellated rods measuring 0.5–0.7 × 1.3–2.7 µm and did not produce spores (see the supplementary figure in IJSEM Online). The growth rates of the isolates at pH 10 in aerobic conditions in the temperature range 10–40 °C were determined using a temperature-gradient incubator (type TN-2612 Bio-Photorecorder; Advantec) by monitoring the OD₆₅₀ value. They grew at temperatures between 10 and 45 °C, their optimum growth temperatures being in the range 30–37 °C. The maximum specific growth rates, $\mu_{\max}$ (h⁻¹), for strain M3^T were as follows: 0.03 at 15 °C, 0.15 at 25 °C, 0.18 at 31 °C, 0.26 at 34 °C, 0.29 at 37 °C and 0.28 at 40 °C. For strain 41A, the rates were as follows: 0.03 at 15 °C, 0.11 at 24 °C, 0.13 at 30 °C, 0.09 at 34 °C, 0.09 at 37 °C and 0.08 at 40 °C. For strain 41C, the rates were as follows: 0.03 at 15 °C, 0.07 at 24 °C, 0.17 at 30 °C, 0.13 at 34 °C, 0.13 at 37 °C and 0.07 at 40 °C.

Analyses of whole-cell fatty acids and isoprenoid quinones were performed as described previously (Yumoto *et al.*, 2002). Peptidoglycan and its hydrolysate were prepared by the method of Okada *et al.* (1992). The amino acid composition was determined using a Hitachi L-8500A automated amino acid analyser. GLC analysis revealed that the cellular fatty acid compositions of strains M3^T, 41A and 41C are as follows (results for the type strain are shown, with the range for the strain in parentheses): C_{10:0}, 20 % (1.5–2.3 %); C_{14:0}, 3.2 % (3.2–5.3 %); C_{14:1}, 3.0 % (2.0–3.3 %); C_{14:1}, 1.2 % (0.9–1.3 %); C_{16:0}, 15.2 % (15.0–26.5 %); C_{16:1}, 4.0 % (3.1–4.0 %); C_{16:1}, 31.1 % (22.8–31.1 %); C_{16:1}, 1.4 % (1.0–1.4 %); C_{18:0}, 3.1 % (3.1–5.9 %); C_{18:1}, 2.2 % (2.1–2.4 %); C_{18:1}, 31.1 % (24.9–31.1 %); and C_{20:0}, 2.5 % (2.5–3.8 %). The isolate contained a large amount of unsaturated fatty acids. The fatty acid compositions of these three isolates were similar to that of *Alkalibacterium olivapovlitis* rather than that of *Alkalibacterium psychrotolerans* and *Marinilactibacillus*

psychrotolerans. However, the $C_{16:0}$ and $C_{18:1}$ contents of our isolates were lower and higher than those of *A. olivapovlenticus*, respectively. Of all the *Alkalibacterium* species, including our isolates, only *A. psychrotolerans* contained more $C_{16:1}9c$ than $C_{16:1}7c$. This means that differences in the lipid biosynthetic pathway may exist in this genus. Isoprenoid quinones could not be detected by HPLC analysis of the concentrated extracted fraction. The purified peptidoglycans of strains M3^T, 41A and 41C contained aspartic acid, ornithine, glutamic acid and alanine, at a molar ratio of 0.6:1.08:1:2.03. These results suggested that the strains possess peptidoglycan type A4 β , Orn-D-Asp. The peptidoglycan amino acid composition is similar to that of *A. olivapovlenticus* NCIMB 13710^T but is not similar to that of *A. psychrotolerans* JCM 12281^T or *M. psychrotolerans* NCIMB 13873^T.

Fermentation products from D-glucose were analysed by HPLC with a TSKgel Oapak column (7.8 mm $\times$ 30 cm $\times$ 2 columns) (Tosoh). The optical purity of L-lactate in the spent medium was determined by HPLC with a TSKgel ENANRIO L1 column (4.6 mm $\times$ 25 cm; Tosoh). The fermentation products of D-glucose (0.5 % in the medium) utilization were determined. Lactate was produced with a yield of 0.68–0.82 [mol (mol glucose)⁻¹] of lactate, 1.25–1.39 [mol (mol glucose)⁻¹] of formate and 0.39–0.71 [mol (mol glucose)⁻¹] of acetate. The optical purity of the L-lactate was found to be 46 %, indicating that the isolates produced DL-lactate.

Bacterial DNA was prepared according to the method of Marmur (1961). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984): the values for strains M3^T, 41A and 41C were in the range 42.6–43.2 mol% – higher than those of the phylogenetically related species *A. olivapovlenticus* (39.7 mol%) and *A. psychrotolerans* (40.6 mol%).

The 16S rRNA gene was amplified using the PCR method with primers A (5'-GGAGAGTTAGATCTTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') for strain M3^T, and primers 20F (5'-AGTTTGATCATGGCTCA-3') and 1541R for strains 41A and 41C, respectively. Although we do not know the reason for this, we were able to amplify objective DNA of M3^T using primer A but not when using primer 20F. The PCR product of approximately 1.5 kb was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100; Applied Biosystems/Hitachi) with a BigDye Termination RR mix, version 3.1 (Applied Biosystems) according to the manufacturer's instructions. Multiple alignments of the sequences were performed and the nucleotide substitution rate (K_{nuc} value) was calculated. A phylogenetic tree was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987), using the CLUSTAL W program (Thompson *et al.*, 1994). Similarity values for sequences were calculated using the GENETYX computer program (Software Development). The 16S rRNA gene sequences of strains M3^T, 41A and 41C were analysed to determine their

phylogenetic positions. The 16S rRNA gene sequences of the three isolates were compared with those of strains belonging to other phylogenetically related taxa. The phylogenetic tree constructed (Fig. 1) and the 16S rRNA gene sequence similarities with 13 related strains (data not shown) showed that the isolates constituted a subcluster with *A. olivapovlenticus* and *A. psychrotolerans* with high bootstrap values and showed high similarities with *Alkalibacterium* spp. The similarity values among the three isolates were 99.4–99.7 %, while the values with respect to the related taxa were less than 97 %. The three strains were placed in phylogenetic positions related to *A. olivapovlenticus* (Ntougias & Russell, 2001), *A. psychrotolerans* (Yumoto *et al.*, 2004) and *M. psychrotolerans* (Ishikawa *et al.*, 2003), all of which are alkaliphiles. The 16S rRNA gene sequence of strain M3^T was found to have the highest similarities with those of the obligate alkaliphiles *A. olivapovlenticus* (96.2 %) and *A. psychrotolerans* (95.8 %), the facultative alkaliphile *M. psychrotolerans* (95.1 %) and the piezotolerant bacterium *Marinilactibacillus piezotolerans* (96.0 %). Strain M3^T was closer phylogenetically to obligate alkaliphiles (genus *Alkalibacterium*) than to facultative alkaliphiles (genus *Marinilactibacillus*). These results demonstrate the obligately alkaliphilic nature of M3^T, which is consistent with the phylogenetic placement of this isolate.

According to the results of 16S rRNA gene sequence analysis, M3^T is closely related to *A. olivapovlenticus* NCIMB 13710^T, *A. psychrotolerans* JCM 12281^T and *M. psychrotolerans* NCIMB 13873^T. *A. olivapovlenticus* NCIMB 13710^T, *A. psychrotolerans* JCM 12281^T and *M. psychrotolerans* NCIMB 13873^T were used as reference strains for DNA–DNA hybridization. These micro-organisms were cultivated using alkali-RCB by the same method. The level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and black microplates (F16 Black Maxisorp; Nage Nunc International). Photobiotin-labelled DNA was prepared using photoprobebiotin (SP-1000; Vector Laboratories). DNA–DNA hybridization indicated that strains M3^T, 41A and 41C belong to the same species (81.1–100 % DNA–DNA similarity) and that strain M3^T differs from *A. psychrotolerans* JCM 12281^T (14.1 % DNA–DNA similarity), *A. olivapovlenticus* NCIMB 13710^T (7.3 % DNA–DNA similarity) and *M. psychrotolerans* NCIMB 13873^T (3.9 % DNA–DNA similarity) when DNA from strain M3^T is used as a probe. Strains M3^T, 41A and 41C are also different from *A. psychrotolerans* JCM 12281^T (10.6–15.1 % DNA–DNA similarity) when DNA from *A. psychrotolerans* JCM 12281^T is used as a probe (see the supplementary table in IJSEM Online). On the basis of DNA–DNA hybridization, 16S rRNA gene sequence similarity and phenotypic and chemotaxonomic characteristics, we conclude that the three isolates belong to the same species.

Strains M3^T, 41A and 41C differed phenotypically and chemotaxonomically from the phylogenetically closely related species *A. olivapovlenticus*, *A. psychrotolerans* and

Fig. 1. Phylogenetic tree of *Alkalibacterium iburiense* strains M3^T, 41A and 41C and other related organisms, derived from 16S rRNA gene sequence data by using the neighbour-joining method. Bootstrap values greater than 500 are shown. Bar, 0.1 K_{nuc} .

M. psychrotolerans, as shown in Table 1. The growth characteristics of the isolates were different from those of *A. psychrotolerans* (Yumoto *et al.*, 2004) and *M. psychrotolerans* (Ishikawa *et al.*, 2003). Although the isolates exhibited phenotypic characteristics similar to those of *A. olivapovliticus*, there were obvious differences in terms of antibiotic sensitivities (chloramphenicol, kanamycin and trimethoprim). The strains belonging to the genus *Alkalibacterium* could be distinguished from other strains belonging to other phylogenetically related genera. They were distinguishable from strains of the genus *Alloiococcus* (Aguirre & Collins, 1992) by catalase reaction, cellular morphology, motility, oxygen requirements, glucose metabolism, growth at pH 7 and DNA G+C content: the genus *Alloiococcus* comprises catalase-positive, non-motile cocci, is aerobic and does not ferment D-glucose, grows at pH 7 and has a DNA G+C content of 44–45 mol%. The strains belonging to the genus *Alkalibacterium* could be distinguished from those of the genus *Dolosigranulum* (Aguirre *et al.*, 1993) by cellular morphology, growth at pH 7 and peptidoglycan type: the latter comprises non-motile ovoid cells, grows at pH 7 and has lysine at position 3 in its peptidoglycan. The

strains belonging to the genus *Alkalibacterium* could be distinguished from those of the genus *Carnobacterium* (Franzmann *et al.*, 1991) by peptidoglycan type and by the production of lactate enantiomer: the genus *Carnobacterium* has Aly-type peptidoglycan, direct cross-linkage and produces mainly L-lactate from D-glucose.

In the present study, we isolated strains belonging to the same species from two different environments. Although strain M3^T (a laboratory contaminant) exhibited 100% DNA–DNA similarity with strain 41A obtained from an indigo sample, the optimum growth temperature, the NaCl concentration range for growth, the growth rate and the fermentation of sugar differed depending on the isolation source. Although we do not know the origin of the contaminant in the case of strain M3^T, it is considered that it might have originated from the ubiquitous environments in which indigo fermentation takes place, because no micro-organism was added artificially to the fermentation. However, we do not know the reason why such an obligately alkaliphilic bacterium exists ubiquitously.

Table 1. Characteristics of *Alkalibacterium iburiense* and related species

+, Positive result; –, negative result; W, weak; ND, not determined.

Characteristic	<i>A. iburiense</i>	<i>A. psychrotolerans</i>	<i>A. olivapovliticus</i>	<i>M. psychrotolerans</i>
Motility	+	+	W	+
Flagella	Peritrichous	Peritrichous	Polar	Peritrichous
Colour of colonies	Pale white	Pale white	Pale white	Pale yellow
Growth pH range	9–12	9–12	≥8, <11.0	6.0–10.0
Optimum pH	9.5–10.5	9.5–10.5	9.0–10.2	8.0–9.5
Growth temp. range (°C)	5–45	5–45	4–37	–1.8 to 40–45
Optimum temp. (°C)	30–37	34	27–32	37–40
Growth NaCl range (%)	0 to 14–16	0–17	0–10	0 to 17.0–20.5
Optimum NaCl concn (%)	3–13	2–12	0–10	2.0–5.0
Specific growth rate μ (h ^{–1})	0.13–0.26	1.67	0.17–0.24	0.6
Antibiotic sensitivity				
Chloramphenicol (2 μ g)	–	ND	+	ND
Kanamycin (2 and 10 μ g)	–	ND	+	ND
Trimethoprim (10 and 25 μ g)	–	ND	+	ND
Peptidoglycan type	A4 β , Orn–D–Asp	A4 β , Orn–D–Glu	A4 β , Orn–D–Asp	A4 β , Orn–D–Glu
Major cellular fatty acids (content above 30%)	C _{16:1} 7c, C _{18:1} 9c	C _{16:0} , C _{16:1} 9c	C _{16:0} , C _{16:1} 7c	C _{16:0} , C _{18:1} 9c
DNA G + C content (mol%)	42.6–43.2	40.6	39.7	34.6–36.2

On the basis of the above results, the isolate was designated a novel species of the genus *Alkalibacterium*, for which the name *Alkalibacterium iburiense* sp. nov. is proposed; the type strain is M3^T. A description of the novel species is given below.

Description of *Alkalibacterium iburiense* sp. nov.

Alkalibacterium iburiense (i.bu.ri.en'se. N.L. neut. adj. *iburiense* from Iburi, the place where the micro-organism was isolated).

Cells are Gram-positive, peritrichously flagellated, straight rods (0.5–0.7 × 1.3–2.7 μ m) and do not produce spores. The intensity of the motility varies depending on the strain. Strains M3^T and 41C exhibit more distinctive motility than strain 41A. Colonies are 2–2.5 mm in size. Both aerobic and anaerobic growth are observed in equal intensities. Colonies are circular, convex and pale white. Catalase, oxidase and aminopeptidase reactions are negative. Growth temperature range is 5–45 or 10–45 °C. Optimum growth temperature is 30–37 °C. NaCl range for growth is 0–16 or 0–14 %. The species grows at pH 9–12 but not at pH 7–8; the optimum pH is 9.5–10.5 at 27 °C. Grows in 0–16 % (w/v) NaCl, with optimum growth at 3–13 % (although the range is wide, the data are reproducible). Grows between 10 and 45 °C, the optimum growth temperature being 30–37 °C. Does not hydrolyse starch or gelatin. Ferments D-glucose, D-arabinose, N-acetylglucosamine and glycogen but does not ferment D-galactose, raffinose, melibiose, myo-inositol, D-mannitol, D-sorbitol or arbutin. Fermentation of D-xylose, maltose, D-mannose, sucrose, D-fructose, L-rhamnose and trehalose is variable (all positive for the

type strain). DL-Lactate is the major end-product from D-glucose. Culture growth is not inhibited by chloramphenicol (2 μ g), kanamycin (2 and 10 μ g), miconazole (2, 10 and 25 μ g), trimethoprim (1.25, 10 and 25 μ g), ketoconazole (25 μ g) and sulfamethoxazole (25 μ g) but is inhibited by ampicillin (10 and 25 μ g), penicillin G (1, 2 and 10 IU) and amoxicillin (10 and 25 μ g). No quinones can be detected. The peptidoglycan type is A4 β , Orn–D–Asp. The major cellular fatty acids are C_{16:0}, C_{16:1}7c and C_{18:1}9c. The DNA G + C content is 42.6–43.2 mol%.

Strain M3^T was isolated from a contaminated culture in alkali broth, and strains 41A and 41C were isolated from a polygonum indigo (*Polygonum tinctorium* Lour.) fermentation liquor obtained from Date city, Iburi, Hokkaido, Japan. The type strain is M3^T (= JCM 12662^T = NCIMB 14024^T).

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