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

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Indigo-reducing, obligately alkaliphilic strains A11^T, F11 and F12 were isolated from indigo fermentation liquor obtained from Tokushima Prefecture, Shikoku, Japan. The isolates grew at pH 9.0–12.3, but not at pH 7.0–8.0. The optimum pH range for growth was 9.5–11.5. They were Gram-negative, facultatively anaerobic, rod-shaped strains with peritrichous flagella. The isolates grew in 0–14 % (w/v) NaCl, with optimum growth at 1–11 %. They grew at temperatures of 15–35 °C with optimum growth at around 20–30 °C. DL-Lactate was the major end product from D-glucose. No quinones were detected. The peptidoglycan type was A4 α , L-Lys (L-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 contents were 47.0–47.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequence data indicated that the isolates belong to the genus *Alkalibacterium*. DNA–DNA hybridization revealed low relatedness values between the isolates and the three phylogenetically most closely related species, *Alkalibacterium olivapovliticus*, *Alkalibacterium psychrotolerans* and *Alkalibacterium iburiense* (<41 %). On the basis of phenotypic characteristics, including hydrolysis of cellulose and fermentation of carbohydrates, and chemotaxonomic characteristics, phylogenetic data and DNA–DNA relatedness data, it is concluded that the isolates merit classification as representatives of a novel species of the genus *Alkalibacterium*, for which the name *Alkalibacterium indicireducens* sp. nov. is proposed. The type strain of this species is A11^T (=JCM 14232^T=NCIMB 14253^T).

Since the isolation of the alkaliphilic lactic acid bacterium *Alkalibacterium olivapovliticus* (Ntougias & Russell, 2001), six species of alkaliphilic lactic acid bacteria with validly published names have been isolated to date (Ishikawa *et al.*, 2003, 2005; Yumoto *et al.*, 2004; Nakajima *et al.*, 2005; Toffin *et al.*, 2005). They have been isolated from artificial environments such as wash water from edible-olive production and fluid obtained from indigo fermentation, as well as natural environments such as living and decomposing marine organisms. This means that this

group of bacteria might be distributed widely in nature and can be isolated by using alkaline media (i.e. pH 10). However, the natural habitat of the isolates obtained from artificial environments is not known. This group contains both obligate and facultative alkaliphiles, and the species are facultative anaerobes, do not produce spores and are halotolerant (grow at >10 % NaCl). The range of DNA G+C contents is 36.2–43.2 mol%.

The traditional method of producing indigo blue dye in Japan is based on fermentation by indigenous alkaliphilic bacteria. Harvested indigo leaves are air-dried and then wetted for appropriate bacterial oxidation under careful moisture control. The obtained product is processed further by microbial reduction under alkaline conditions (pH >10), at which point the original, insoluble, oxidized form of indigo is converted to a soluble, reduced form. If indigo-reducing bacteria can be isolated for use as

Abbreviation: TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains A11^T, F11 and F12 are AB268549–AB268551, respectively.

A supplementary table showing the fatty acid profiles of *Alkalibacterium indicireducens* sp. nov. and related species is available with the online version of this paper.

inoculants, it will be easy to initiate proper fermentation. Revival of the traditional method of producing indigo dye would reduce the use of chemical reagents, which are burdens on the natural environment. Indigo-reducing bacteria have been isolated by Takahara & Tanabe (1960) and Padden *et al.* (1999) and have been identified as *Bacillus* sp. and *Clostridium isatidis*, respectively. However, the bacterium isolated by Takahara & Tanabe (1960) is unfortunately no longer available. In addition to these isolates, the indigo-reducing bacteria *Alkalibacterium psychrotolerans* and *Alkalibacterium iburiense* have also been isolated recently from indigo fermentation liquor obtained from Date City, Iburi, Hokkaido, Japan (Yumoto *et al.*, 2004; Nakajima *et al.*, 2005).

In this study, halotolerant, obligately alkaliphilic lactic acid bacterial strains that can reduce indigo at high pH were isolated from indigo fermentation liquor samples obtained from Tokushima Prefecture, Shikoku, Japan, and characterized. Results of phenotypic and chemotaxonomic characterization, phylogenetic analysis based on 16S rRNA gene sequences, and DNA–DNA hybridization showed that the isolates merit classification as representatives of a novel species of the genus *Alkalibacterium*, *Alkalibacterium indicireducens* sp. nov.; the type strain is A11^T (=JCM 14232^T=NCIMB 14253^T).

Fermented polygonum indigo (*Polygonum tinctorium* Lour.) liquor samples were obtained from Tokushima City (34° 04' N 134° 31' E) and Aizumi-cho, Itano-gun (34° 07' N 134° 29' E), Tokushima Prefecture, Shikoku, Japan, on 30–31 January 2003. Upon reaching the laboratory, 5 ml indigo 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 carried out five times using the same medium. The enrichment culture thus obtained was inoculated on a reinforced clostridial agar (RCA; Sigma) plate containing 100 mM NaHCO₃/Na₂CO₃ buffer pH 10 (alkali RCA) and incubated in an argon-exchanged jar. Three strains that were positive for indigo reduction were identified during the screening process. One strain, A11^T, was isolated from a sample obtained from Tokushima City, and two strains, F11 and F12, were isolated from a sample obtained from Aizumi-cho, Itano-gun. After 48 h cultivation, single colonies were selected and re-isolated 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 isolates to reduce indigo. Cells for chemotaxonomic analysis were harvested in the late-exponential phase after cultivation with reciprocal shaking

(130 strokes min⁻¹) at 27 °C in reinforced clostridial broth (RCB) (clostridial nutrient medium; Fluka BioChemika) containing 100 mM NaHCO₃/Na₂CO₃ buffer, pH 10 (alkali RCB). NaHCO₃/Na₂CO₃ buffer 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 various pH values were performed using PYA medium containing 100 mM NaH₂PO₄/Na₂HPO₄ buffer (pH 7.0–8.0), 100 mM NaHCO₃/Na₂CO₃ buffer (pH 9.0–10.0), Na₂HPO₄/NaOH buffer (pH 11.0) or KCl/NaOH buffer (pH 12.0–12.3). Anaerobic growth was tested in PYA broth (pH 10) by substituting air with argon. Other physiological and biochemical characteristics were examined according to the methods of Yumoto *et al.* (1998). Xylan and cellulose hydrolysis was assessed on alkali RCA containing 1 % substrate and judged by the method of Teather & Wood (1982). Antibiotic sensitivity was investigated by placing a small amount of antibiotic impregnated in a paper disc on plates of alkali RCA medium. For the observation of negatively stained cells by transmission electron microscopy (TEM), cells were grown on alkali RCA. TEM preparation and observation were performed as described previously (Yumoto *et al.*, 2001). The growth rates of the isolates at pH 10 under aerobic conditions in the temperature range 10–40 °C were determined by using a temperature-gradient incubator (Bio-Photorecorder type TN-2612; Advantec) by monitoring OD₆₅₀. The isolates can be differentiated from previously reported species belonging to the genus *Alkalibacterium* by the characteristics listed in Table 1, such as hydrolysis of cellulose and fermentation of carbohydrates.

Bacterial DNA was prepared according to the method of Marmur (1961). DNA base composition was determined by the method of Tamaoka & Komagata (1984) and the results are shown in Table 1. 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). Amino acid composition was determined by using a Hitachi L-8500 automated amino acid analyser. GLC analyses revealed that the cellular fatty acid compositions (%) of strains A11^T, F11 and F12 are as follows (results are for the type strain and values in parentheses indicate the range found for the three strains): C_{10:0}, 0.7 (0.0–0.7); C_{14:0}, 9.3 (2.7–9.3); C_{14:15c}, 1.4 (0.5–1.4); C_{14:17c}, 0.9 (0.2–0.9); C_{16:0}, 42.0 (40.0–52.3); C_{16:15c}, 2.4 (1.7–2.4); C_{16:17c}, 27.4 (15.5–27.4); C_{16:19c}, 2.7 (0.3–2.7); C_{18:0}, 2.3 (2.3–8.6); C_{18:19t}, 0.5 (0.5–1.4); C_{18:19c}, 11.5 (11.5–23.1); C_{20:0}, 0.3 (0.3–5.5). Each of the isolates

Table 1. Differential characteristics of strains A11^T, F11 and F12 and related *Alkalibacterium* species

Strains: 1, A11^T; 2, F11; 3, F12; 4, *A. iburiense* strains M3^T, 41A and 41C; 5, *A. psychrotolerans* JCM 12281^T; 6, *A. olivapovlenticus* NCIMB 13710^T. +, Positive; –, negative; w, weakly positive; d, strain-dependent. Data for *A. iburiense*, *A. psychrotolerans* and *A. olivapovlenticus* are from Nakajima *et al.* (2005), Yumoto *et al.* (2004) and Ntougias & Russell (2001), respectively.

Characteristic	1	2	3	4	5	6
Gram stain	–	–	–	+	+	+
Flagella	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Polar
pH range for growth	9.0–12.3	9.0–12.3	9.0–12.3	9–12	9–12	≥8, <11.0
Optimum pH	9.5–11.5	9.5–11.5	9.5–11.5	9.5–10.5	9.5–10.5	9.0–10.2
Temperature range for growth (°C)	15–40	15–40	18–37	5–45	5–45	4–37
Optimum temperature (°C)	20–30	20–30	20–30	30–37	34	27–32
NaCl range for growth (%)	0–15	0–14	0–14	0 to 14–16*	0–17	0–10
Optimum NaCl concentration (%)	1–11	1–11	1–11	3–13	2–12	0–10
Specific growth rate, $\mu_{\max}$ (h ^{–1})	0.36	0.32	0.22	0.13–0.26	1.67	0.17–0.24
Hydrolysis of cellulose†	+	+	+	–	–	–
Fermentation of:						
D-Arabinose	–	–	–	+	+	–
D-Xylose	–	–	–	d	+	–
Maltose	–	–	–	d	+	+
Trehalose	–	–	–	d	–	+
D-Mannose	–	–	–	d	w	+
Melibiose	–	–	–	–	–	+
Antibiotic sensitivity						
Chloramphenicol (2 µg)	–	–	–	–	–	+
Kanamycin (2 and 10 µg)	–	–	–	–	–	+
Trimethoprim (10 and 25 µg)	–	–	–	–	+	+
Peptidoglycan type	A4 α , L-Lys (L-Orn)–D-Asp	A4 α , L-Lys (L-Orn)–D-Asp	A4 α , L-Lys (L-Orn)–D-Asp	A4 α , L-Lys–D- Asp	A4 α , L-Lys (L-Orn)–D-Asp	A4 β , L-Orn–D- Asp
DNA G + C content (mol%)	47.1	47.0	47.8	42.6–43.2	40.6	39.7

*Upper pH limit varies depending on strain.

†Hydrolysis of cellulose was tested in this study.

contained a large amount of unsaturated fatty acids. The fatty acid compositions of these three isolates were similar to those of *A. iburiense* and *A. olivapovlenticus*, rather than to that of *A. psychrotolerans* (see Supplementary Table S1, available in IJSEM Online). However, the C_{16:0} contents of the isolates were higher than those of other *Alkalibacterium* species. Of members of the genus *Alkalibacterium*, including the novel isolates, only *A. psychrotolerans* contained a larger amount of C_{16:1}9c than C_{16:1}7c. Isoprenoid quinones could not be detected by HPLC analysis from the concentrated extracted fraction. The purified peptidoglycans of strains A11^T, F11 and F12 contained D-Asp, L-Orn + L-Lys, L-Glu and L-Ala in the molar ratio 0.60–0.64:0.92–1.11:1:1.7–2.13. These results indicated that the strains possess the A4 α , L-Lys (L-Orn)–D-Asp type of peptidoglycan. The amino acid composition of the peptidoglycan is similar to those of other species belonging to the genus *Alkalibacterium* [*A. psychrotolerans*, L-Lys (L-Orn)–D-Asp; *A. iburiense*, L-Lys–D-Asp].

Fermentation products from D-glucose [0.5 % (w/v) in the medium] were analysed by HPLC with TSKgel OApak-A column (7.8 mm × 30 cm × 2 columns) (Tosoh). The

optical purity of L-lactate in the spent medium was determined by HPLC with a TSKgel ENANTIO L1 column (4.6 mm × 25 cm × 2 columns) (Tosoh). Lactate, formate and acetate were produced from D-glucose with yields of 1.51–1.75, 0.82–1.07 and 0.14–0.36 mol (mol glucose)^{–1}, respectively. The optical purity of the L-lactate was 46–47 %, indicating that the isolate produced DL-lactate.

The 16S rRNA gene was amplified by PCR with primers 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCC-3') for strain A11, 20F (5'-AGTTTGATCATGGCTCA-3') and 1541R for strain F11, and 105F (5'-GCGGACGGGTGAGTAACAC-3') and 1541R for strain F12. Although the reason is unclear, objective DNA in each strain could be amplified by using the primer sets described above. The PCR product of approximately 1.5 kb was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100) 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 program CLUSTAL W (Thompson *et al.*, 1994). Similarity values for sequences were calculated using the GENETYX computer program (Software Development). The 16S rRNA gene sequences of strains A11^T, F11 and F12 were analysed to determine their phylogenetic positions and compared with those of strains belonging to other phylogenetically related taxa. The phylogenetic tree constructed, including closely related taxa (Fig. 1), showed that the isolates constituted a subcluster with *A. olivapovliticus*, *A. psychrotolerans* and *A. iburiense* and showed high similarities to these *Alkalibacterium* species. The similarity values among the three isolates were 99.2–99.6 %, whereas those of the isolates to related strains were <98.6 %. The three strains were placed in phylogenetic positions related to *A. olivapovliticus* (Ntougias & Russell, 2001), *A. psychrotolerans* (Yumoto *et al.*, 2004) and *A. iburiense* (Nakajima *et al.*, 2005), which are all lactate-producing alkaliphiles. The 16S rRNA gene sequence of strain A11^T had highest similarities to those of the type strains of the obligate alkaliphiles *A. olivapovliticus* (98.5 %) and *A. psychrotolerans* (96.8 %) and to *A. iburiense* (96.2–96.4 %;

all three known strains, including the type strain), as well as to the sequences of the type strains of the facultative alkaliphile *Marinilactibacillus psychrotolerans* (95.1 %) and the piezotolerant bacterium *Marinilactibacillus piezotolerans* (97.0 %).

On the basis of the results of 16S rRNA gene sequence analysis, *A. olivapovliticus* NCIMB 13710^T, *A. psychrotolerans* JCM 12281^T and *A. iburiense* JCM 12662^T were used as reference strains for DNA–DNA hybridization. These micro-organisms and the three isolates were cultivated by using 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. DNA–DNA hybridization results indicated that strains A11^T, F11 and F12 belong to the same species (87–97 % DNA–DNA relatedness) and that strain A11^T differed from *A. psychrotolerans* JCM 12281^T (40 % DNA–DNA relatedness), *A. olivapovliticus* NCIMB 13710^T (21 % DNA–DNA relatedness) and *A. iburiense* JCM 12662^T (34 % DNA–DNA relatedness) when DNA from strain A11^T was used as a probe. It is concluded that the three isolates belong to the same species and are different from previously reported *Alkalibacterium* species.

In addition to the results of the phylogenetic analysis and the DNA–DNA hybridization experiment, strains A11^T, F11 and F12 differed in terms of growth, phenotypic characteristics and chemotaxonomic characteristics from reported *Alkalibacterium* species, as shown in Table 1. On the basis of the above results, the isolates represent a novel species of the genus *Alkalibacterium*, for which the name *Alkalibacterium indicireducens* sp. nov. is proposed; the type strain is A11^T.

Description of *Alkalibacterium indicireducens* sp. nov.

Alkalibacterium indicireducens (in.di.ci.re.du'cens. L. n. *indicum* indigo; L. part. adj. *reducens* bringing or leading back, used to mean 'reducing'; N.L. part. adj. *indicireducens* indigo-reducing).

Cells are Gram-negative, peritrichously flagellated, straight rods (0.4–1.2 × 1.7–3.7 µm) that do not produce spores. Strains of this species have various degrees of motility; strain F11 exhibits more distinctive motility than strains A11^T and F12. Colonies are 0.5–2.0 mm, circular, convex and pale white. Similar-sized colonies are produced during both aerobic and anaerobic growth. Catalase, oxidase and aminopeptidase reactions are negative. DL-Lactate is the major end product from D-glucose. Growth temperature range is 15–40 °C (strains A11^T and F11) or 18–37 °C (strain F12); optimum is 20–30 °C. NaCl range for growth is 0–15 % (strain A11^T) or 0–14 % (strains F11 and F12); optimum is 1–11 % NaCl. Grows at pH 9.0–12.3; optimum growth occurs at pH 9.5–11.5. Hydrolyses starch, xylan and cellulose, but not casein or gelatin. Ferments D-glucose and sucrose weakly. Fermentation of D-fructose is variable (positive for the type strain). Fermentation of D-arabinose,

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence data of *A. indicireducens* strains A11^T, F11 and F12 and other related organisms by using the neighbour-joining method. Numbers at branching points indicate bootstrap values (from 1000 replicates). Bar, 0.1 K_{nuc} .

D-xylose, D-galactose, inositol, maltose, raffinose, trehalose, D-mannose, melibiose, L-rhamnose and sorbitol is negative. Growth is not inhibited by chloramphenicol (2 and 10 µg), kanamycin (2 and 10 µg), miconazole (2 µg), trimethoprim (1.25, 10 and 25 µg), ketoconazole (25 µg) or sulfamethoxazole (25 µg), but is inhibited by ampicillin (2, 10 and 25 µg), penicillin G (1, 2 and 10 IU) and amoxycillin (10 and 25 µg). No quinones can be detected. The major cellular fatty acids are C_{16:0}, C_{16:17c} and C_{18:19c}. The peptidoglycan type is A4 α , L-Lys (L-Orn)-D-Asp. The DNA G + C content is 47.0–47.8 mol%.

The type strain is A11^T (=JCM 14232^T=NCIMB 14253^T); F11 and F12 are reference strains. All strains were isolated from a polygonum indigo (*Polygonum tinctorium* Lour.) fermentation liquor. A11^T was isolated from a sample obtained from Tokushima City and F11 and F12 were isolated from a sample obtained from Aizumi-cho, Itanogun, Japan.

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