Alkalibacterium psychrotolerans sp. nov., a psychrotolerant obligate alkaliphile that reduces an indigo dye

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A psychrotolerant, obligately alkaliphilic bacterium, IDR2-2^T, which is able to reduce indigo dye was isolated from a fermented polygonum indigo (*Polygonum tinctorium* Lour.) produced in Date, Hokkaido, using a traditional Japanese method. The isolate grew at pH 9–12 but not at pH 7–8. It was a Gram-positive, facultatively anaerobic, straight rod-shaped bacterium with peritrichous flagella. The isolate grew in 0–17 % (w/v) NaCl but not at NaCl concentrations higher than 18 % (w/v). Its major cellular fatty acids were C_{14:0}, C_{16:0}, C_{16:1}9c and C_{18:1}9c, and its DNA G+C content was 40.6 mol%. DL-Lactic acid was the major end-product from D-glucose. No quinones could be detected. The peptidoglycan type was A4β, Orn–D–Glu. A phylogenetic analysis based on 16S rRNA gene sequence data indicated that strain IDR2-2^T is a member of the genus *Alkalibacterium*. DNA–DNA hybridization revealed low relatedness (less than 25 %) between the isolate and two phylogenetically related strains, *Alkalibacterium olivapovliticus* and *Marinilactibacillus psychrotolerans*. On the basis of phenotypic characteristics, phylogenetic data and DNA–DNA relatedness data, the isolate merits classification as a novel species, for which the name *Alkalibacterium psychrotolerans* sp. nov. is proposed. The type strain is IDR2-2^T (=JCM 12281^T=NCIMB 13981^T).

Alkaliphilic micro-organisms have been isolated in order to investigate their diversity, taxonomy and physiological adaptation to high pH values, and to utilize their enzymes industrially (Horikoshi, 1991; Krulwich *et al.*, 2001; Yumoto, 2002, 2003). Although considered to be extremophiles, alkaliphilic micro-organisms are distributed not only in unique places such as alkaline soda lakes (Duckworth *et al.*, 1996) but also in ordinary soils or faeces (Horikoshi, 1991). In this respect, alkaliphilic micro-organisms are different from other extremophiles. This might be due to the fact that niches for alkaliphiles are relatively small, e.g. the intestines of insects (high pH), and are distributed throughout natural ordinary environments (Thongaram *et al.*, 2003). Alkaliphilic bacteria can be either facultative or obligate (Krulwich & Guffanti, 1989). Bacteria of the former type can grow at neutral pH, whereas the latter cannot. Numerous alkaliphilic micro-organisms have been isolated,

including members of the *Archaea* and *Bacteria*. Both Gram-positive and Gram-negative alkaliphilic bacteria have been isolated. Alkaliphilic bacteria generally consist of not only aerobic or facultatively anaerobic micro-organisms but also obligate anaerobes; they are divided into psychroalkaliphiles, mesoalkaliphiles, thermoalkaliphiles and haloalkaliphiles (Pikuta *et al.*, 2000; Yumoto *et al.*, 2001, 2002; Belduz *et al.*, 2003).

Traditionally, indigo blue dye has been produced from a Japanese indigo plant. The indigo leaves are air-dried and then appropriately wetted to increase aerobic microbial oxidation activity. The product is fermented under alkaline conditions (pH values above 10), at which point the original insoluble oxidized form of indigo is converted, by the micro-organisms, to the soluble reduced form. The use of fermentation to produce indigo declined around 1960 because of difficulties in the production and maintenance processes; since then, the dye has been produced by a chemical process. Recently, traditional methods have been re-evaluated, because they produce better products, in terms of colour, than do the recently invented methods.

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IDR2-2^T is AB125938.

Indigo-reducing bacteria have been isolated by Takahara & Tanabe (1960) and Padden *et al.* (1999) and identified as *Bacillus* sp. and *Clostridium isatidis*, respectively.

In this study, a halotolerant, psychrotolerant, obligately alkaliphilic micro-organism that can reduce indigo at high pH values was isolated. Phenotypic characterization, phylogenetic analysis based on 16S rRNA gene sequences and DNA–DNA hybridization showed that the isolate merits classification as a novel *Alkalibacterium* species.

Fermented polygonum indigo (*Polygonum tinctorium* Lour.) samples were obtained from Date, Hokkaido, Japan (42° 42' N, 140° 42' E). Fermentation liquor (5 ml) was inoculated into 100 ml 0.01 % indigo-containing peptone/yeast extract/alkaline buffer (PYA) 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·nH₂O, 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 continued 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 at pH 10 (alkali-RCA) and incubated in an argon gas exchange jar. After 48 h cultivation, single colonies were picked up 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 isolate 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; Sigma) containing 100 mM NaHCO₃/Na₂CO₃ (alkali-RCB). Alkali solution for media used in this study was sterilized separately by autoclaving. *Alkalibacterium olivapovlenticus* NCIMB 13710^T and *Marinilactibacillus 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.

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 for reproducibility of results. Acid production from carbohydrates was determined according to the method of Hugh & Leifson (1953), using thymol blue, 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 replacing air with argon gas. Other physiological and biochemical characteristics were examined according to the methods of Yumoto *et al.* (1998) and as described by Barrow & Feltham (1993). The aerobic

growth rate, at pH 10, of the isolate in the temperature range 10–40 °C was determined using a temperature-gradient incubator (Bio-Photorecorder, type TN-2612; Advantec).

For observation of negatively stained cells by transmission electron microscopy, cells were grown on alkali-RCA; preparation and observation were performed as described previously (Yumoto *et al.*, 2002).

Whole-cell fatty acid analysis and isoprenoid quinone determination were performed as described previously (Yumoto *et al.*, 2001). 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.

Fermentation products from D-glucose were analysed by HPLC with a TSKgel Oapak (Tosoh) column (7.8 mm × 30 cm × 2). The optical purity of L-lactate in the spent medium was determined by HPLC with a TSKgel ENANRIO L1 (Tosoh) column (4.6 mm × 25 cm).

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 level of DNA–DNA relatedness was determined fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and black microplates.

The 16S rRNA gene sequence corresponding to positions 27–1519 in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978) was amplified by a PCR with primers 20F (AGTTTGATCATGGCTCA) and 1541R (AAGGAGGTGATCCAGCC). The PCR product (approx. 1.5 kb) was sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (PRISM 3100; Applied Biosystems). 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).

One strain was isolated using RCA (pH 10). The colonies of strain IDR2-2^T on an alkali-RCA plate were circular, convex and pale white; IDR2-2^T cells were Gram-positive, peritrichously flagellated rods measuring 0.4–0.9 × 0.7–3.1 µm and did not produce spores (Fig. 1).

The physiological and biochemical characteristics of the isolate are given in the species description. The isolate grew equally well at all pH values in the range 9–12, but did not grow at pH 7–8; optimum growth was at pH 9.5–10.5. It is facultatively anaerobic. It grows between 5 and 45 °C, the optimum growth temperature being around 34 °C. The maximum specific growth rates, *μ*_{max} (h⁻¹), for strain IDR2-2^T were 0.4 at 10 °C, 0.5 at 15 °C, 1.25 at

Fig. 1. Transmission electron micrograph of a negatively stained cell of *A. psychrotolerans* sp. nov. IDR2-2^T. Bar, 1 µm.

25 °C, 1.43 at 30 °C, 1.67 at 34 °C, 1.55 at 37 °C and 0.53 at 40 °C.

The end-products of glucose utilization were determined. Lactate was produced with a yield of 80–88 % relative to the amount of added D-glucose (0.5 %). The optical purity of the L-lactate was found to be 52 %, indicating that the bacterium produced DL-lactate.

GLC analysis revealed that the fatty acids of IDR2-2^T are C_{10:0} (0.7 %), C_{14:0} (10.9 %), C_{16:0} (37.7 %), C_{16:17c} (2.2 %), C_{16:19c} (34.7 %), C_{18:0} (1.5 %) and C_{18:19c} (10.3 %). The isolate contained a large amount of unsaturated fatty acids. The fatty acid composition of IDR2-2^T was similar to that of *A. olivapovlenticus* rather than that of *M. psychrotolerans*.

Isoprenoid quinones could not be detected by HPLC analysis of the concentrated extracted fraction. The purified peptidoglycan of IDR2-2^T contained glutamic acid, alanine and ornithine in a molar ratio of 1:1.2:0.8. From the results, it was suggested that the strain possessed peptidoglycan of the A4β type (Orn–D–Glu). The amino acid composition in the peptidoglycan is similar to that of *M. psychrotolerans* NCIMB 13873^T rather than that of *A. olivapovlenticus* NCIMB 13710^T.

A 1492-base 16S rRNA gene sequence of IDR2-2^T was compared with those of strains belonging to other phylogenetically related taxa. The phylogenetic tree constructed using the neighbour-joining method (Fig. 2) and 16S rRNA gene sequence similarities with 12 related strains (data not shown) showed that IDR2-2^T is a member of the genus *Alkalibacterium*. The values for similarity to the related taxa were less than 97 %. IDR2-2^T was placed in a related

Fig. 2. Phylogenetic tree derived from 16S rRNA gene sequence data of *A. psychrotolerans* sp. nov. IDR2-2^T and other related organisms using the neighbour-joining method. Numbers indicate bootstrap values greater than 500. Bar, 0.1 K_{nuc} .

phylogenetic position with respect to *A. olivapovlenticus* (Ntougias & Russell, 2001) and *M. psychrotolerans* (Ishikawa *et al.*, 2003), which are both alkaliphiles. The highest similarities, in terms of 16S rRNA gene sequence, for IDR2-2^T were with the obligate alkaliphile *A. olivapovlenticus* (96.9 %) and the facultative alkaliphile *M. psychrotolerans* (94.6 %). IDR2-2^T exhibited higher levels of 16S rRNA gene sequence similarity with the obligate alkaliphile than with the facultative alkaliphile. These results demonstrate that the obligately alkaliphilic nature of IDR2-2^T is consistent with the phylogenetic placement of this isolate.

The DNA G + C content of IDR2-2^T was 40.6 mol%, which is close to that of *A. olivapovlenticus* (39.7 mol%).

According to the results of 16S rRNA gene sequence analysis, IDR2-2^T is closely related to *A. olivapovlenticus* and *M. psychrotolerans*. DNA–DNA hybridization indicates that IDR2-2^T is different from *A. olivapovlenticus* (24.3 % DNA–DNA relatedness) and *M. psychrotolerans* (7.6 % DNA–DNA relatedness).

IDR2-2^T differed phenotypically and chemotaxonomically from its phylogenetically close relatives *A. olivapovlenticus* and *M. psychrotolerans*, as shown in Table 1. The strain could be distinguished from other strains belonging to other phylogenetically related genera: it is distinguished from the genus *Alloiococcus* by cellular morphology, motility, metabolism of glucose, catalase reaction and the DNA G + C content (the genus *Alloiococcus* is catalase-positive, comprises non-motile cocci, does not ferment glucose and the DNA G + C content is 44–45 mol%); from the genus *Dolosigranulum* by cellular morphology and the peptidoglycan type (the genus *Dolosigranulum* has

Table 1. Characteristics of *Alkalibacterium psychrotolerans* sp. nov. and related species

All three strains were positive for fermentation of D-glucose and maltose and negative for fermentation of raffinose, *myo*-inositol and sorbitol. Characteristics of *A. olivapovliticus* and *M. psychrotolerans* were cited from Ntougias & Russell (2001) and Ishikawa *et al.* (2003), respectively. +, Positive; –, negative; W, weakly positive; ND, not determined.

Characteristic	<i>A. psychrotolerans</i> sp. nov. IDR2-2 ^T	<i>A. olivapovliticus</i> WW2-SN4a ^T	<i>M. psychrotolerans</i> M13-2 ^T
Motility	+	W	+
Flagella	Peritrichous	Polar	Peritrichous
Colour of colonies	Pale white	Pale white	Pale yellow
pH for growth:			
Range	9–12	≥8, <11·0	6·0–10·0
Optimum	9·5–10·5	9·0–9·4	8·5–9·0
Temperature (°C) for growth:			
Range	5–45	4–35	–1·8 to 40–45
Optimum	34	27–32	37–40
NaCl (% w/v) for growth:			
Range	0–17	0–15	0–17·0–20·5
Optimum	2–12	3–5	2·0–3·75
Fermentation of sugars			
D-Arabinose	+	–	+
D-Xylose	+	–	–
D-Galactose	–	–	+
D-Rhamnose	–	–	+
Lactose	–	–	W
Melibiose	–	–	+
Sucrose	–	+	+
Mannitol	–	–	+
Inulin	–	ND	+
Peptidoglycan type	A4β, Orn–D-Glu	A4β, Orn–D-Asp	A4β, Orn–D-Glu
Major cellular fatty acids (>30%)	C _{16:0} , C _{16:1}	C _{16:0} , C _{16:1}	C _{15:0} , C _{18:0}
DNA G+C content (mol%)	40·6	39·7	36·2

non-motile, ovoid cells and has lysine at position 3 in the peptidoglycan); and from the genus *Carnobacterium* by peptidoglycan type and in the main enantiomer of lactate produced (the genus *Carnobacterium* has peptidoglycan of the A1γ type, direct cross-linkage and produces mainly L-lactate from D-glucose).

On the basis of the above results, the isolate was designated as representing a novel species, for which the name *Alkalibacterium psychrotolerans* sp. nov. is proposed, with IDR2-2^T as the type strain.

Description of *Alkalibacterium psychrotolerans* sp. nov.

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

Cells are Gram-positive, peritrichously flagellated straight rods (0·4–0·9 × 0·7–3·1 μm) and do not produce spores. Grows equally well aerobically and anaerobically. Colonies are circular, convex and pale white. Catalase and oxidase reactions are negative. The species grows at pH 9–12 but not at pH 7–8; optimum growth is at pH 9·5–10·5 at 27 °C.

It grows in 0–17% (w/v) NaCl, with 2–12% (w/v) as the optimum concentration. It grows at temperatures between 5 and 45 °C, the optimum growth temperature being around 34 °C. It does not hydrolyse starch or gelatin. Ferments D-arabinose, D-xylose, D-glucose and maltose. Does not ferment melibiose, raffinose, *myo*-inositol, mannitol, erythritol, sorbitol, xylitol, adonitol, dulcitol, sucrose, galactose, inulin or rhamnose. DL-Lactic acid is the major end-product from D-glucose. No quinones can be detected. The peptidoglycan type is A4β, Orn–D-Glu. The major cellular fatty acids are C_{14:0}, C_{16:0}, C_{16:1}9c and C_{18:1}9c. The DNA G+C content is 40·6 mol%.

The type strain, strain IDR2-2^T (=JCM 12281^T=NCIMB 13981^T), was isolated from polygonum indigo (*Polygonum tinctorium* Lour.) fermentation liquor obtained from Date, Hokkaido, Japan.

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