**Paralkalibacillus indicireducens** gen., nov., sp. nov., an indigo-reducing obligate alkaliphile isolated from indigo fermentation liquor used for dyeing

Kikue Hirota,¹ Masatoshi Nishita,² Hidetoshi Matsuyama² and Isao Yumoto¹,³,*

Abstract

Obligately alkaliphilic, indigo-reducing strains, designated Bps-1ᵀ, Bps-2 and Bps-3, were isolated from an indigo fermentation liquor used for dyeing, which was produced from *sukumo* (composted *Polygonum* indigo leaves) obtained from a craft centre in Data City, Hokkaido, Japan, by using medium containing cellulase-treated *sukumo*. The 16S rRNA gene sequence phylogeny suggested that Bps-1ᵀ has a distinctive position among the alkaliphilic species of the genus *Bacillus*, with its closest neighbours being *Bacillus pseudofirmus* DSM 8715ᵀ, *Bacillus indiannensis* DSM 26864ᵀ and *Bacillus alcalophilus* DSM 485ᵀ (96.1, 95.8 and 95.5 % 16S rRNA gene sequence similarities, respectively). The 16S rRNA sequence of strain Bps-1ᵀ was identical to those of strains Bps-2 and Bps-3. Cells of the novel isolate were Gram-stain-positive and were facultatively anaerobic straight rods that were motile by means of a pair of flagella (subpolar and centre sides). Spherical endospores were formed in the terminal position. Strain Bps-1ᵀ grew between 18 and 40 °C with optimum growth at 33 °C. The isolate grew in the pH range 8–11, with optimum growth at pH 9–10. The isoprenoid quinone detected was menaquinone-7 (MK-7), and the DNA G+C content was 40.3 %. The whole-cell fatty acid profile (>10 %) mainly consisted of anteiso-C₁₅:₀, iso-C₁₅:₀ and C₁₆:₀. On the basis of the phenotypic, chemotaxonomic and phylogenetic data, the isolates represent a novel species of a novel genus, for which the name *Paralkalibacillus indicireducens* gen. nov., sp. nov. is proposed. The type strain of this species is Bps-1ᵀ (JCM 31808²=NCIMB 15080²), with strains Bps-2 and Bps-3 representing additional strains of the species.

For the preparation of the dye source in indigo fermentation fluid used for dyeing, *sukumo*, which is composted indigo leaves, is first prepared after the harvest of indigo leaves by the traditional Japanese method. Prepared *sukumo* is then used in the next step of fermentation in liquid in order to solubilize the indigo by exploiting the reducing capacity of microorganisms. At the initiation of fermentation, *sukumo* is mixed with approximately 60–80 °C wood ash extract (pH 10.5–11), and fermentation is initiated by keeping the pH above 10.5 at 25–40 °C. The fermentation fluid is stirred once or twice per day under these anaerobic, alkaline conditions, the isolation and identification of microorganisms is indispensable. The aerotolerant anaerobic bacteria *Alkalibacterium* spp. [2–4] and *Amphibacillus* spp. [5, 6] have been isolated and identified as indigo-reducing bacteria. *Oceanobacillus indicireducens*, a bacterial strain that metabolizes oxygen, but lacks menaquinone [7] has also been isolated as an indigo-reducing bacterium. In addition, novel genera and species of indigo-reducing and facultatively anaerobic bacteria, such as *Fermentibacillus polygoni* and *Polygonibacillus indicireducens* have recently been isolated [8, 9].

Microbiota in indigo-reducible fermentation fluid exhibit differences arising from variations in materials, management procedures and fermentation age [10]. In addition, it is expected that many currently unrecognised species exist.

**Author affiliations:** ¹Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8571, Japan; ²School of Biological Science and Engineering, Tokai University, Minamisawa, Minami-ku, Sapporo 005-8601, Japan; ³Laboratory of Molecular Environmental Microbiology, Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan.

*Correspondence: Isao Yumoto, i.yumoto@aist.go.jp*

**Keywords:** *Paralkalibacillus indicireducens*; alkaliphilic; indigo fermentation liquor; indigo reduction.

**Abbreviations:** DAP, Diaminopimelic acid; SE, Sukumo extract; SWE, Sukumo/wheat bran extract.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains Bps-1ᵀ, Bps-2 and Bps-3 are LC197841, LC197869 and LC197842, respectively.

One supplementary table and four supplementary figures are available with the online Supplementary Material.
in the fluid due to the many factors involved in natural fermentation because of the variable nature of the natural fermentation process. To increase the possibility of finding novel indigo-reducing bacteria, an appropriate procedure would be the utilization of a suitable medium for their isolation.

In the present study, indigo-reducing, obligately alkaliphilic strains were isolated from a medium containing sukumo and wheat bran hydrolysates from an indigo fermentation liquor used for dyeing. Phenotypic and chemotaxonomic characterization, phylogenetic analysis, based on the 16S rRNA gene sequences, and DNA–DNA hybridization showed that the isolates merit classification as a novel species of a novel genus.

Indigo fermentation liquor was obtained from an indigo fermentation craft centre in Date City, Hokkaido, Japan (42°28′14.977″N, 140°52′31.648″E). To degrade the substrate, 30 g of wheat bran or sukumo was mixed with 0.5 g cellulase (Amano enzyme), and this was followed by incubation in 100 mM acetate buffer (pH 5.0) at 50°C for 20 h in a water bath. The resulting fluid was adjusted to pH 7 and filtered with filter paper (pore size: 4 µm). An aliquot of the sample (0.5 ml) was diluted 10^5 times with a series of 4.5 ml aliquots of physiological saline and inoculated into SE (sukumo/extract: pH 10) and SWE (sukumo/wheat bran/extract: pH 10) [11]. SE medium was composed of 15 ml sukumo hydrolysate, 2 g indigo carmine and 15 g gellan gum in 885 ml distilled water. SWE medium was composed of 15 ml sukumo hydrolysate, 15 ml wheat bran hydrolysate, 2 g indigo carmine and 15 g gellan gum in 870 ml distilled water. A ten-fold concentration of alkaline solution (10% w/v NaHCO_3) was separately sterilized and mixed with each autoclaved medium at a 10% ratio (the final concentration of NaHCO_3 was 1%, w/v). The inoculated media were incubated at 27°C for 2 days under an N_2 gas-exchange anaerobic jar to suppress the growth of obligately aerobic micro-organisms. Two strains (Bps-1^T and Bps-3) and one strain (Bps-2) were isolated from SE and SWB media, respectively, as indigo-reducing bacteria. *Bacillus pseudofermus* JCM 8715^T, *Bacillus lindanensis* 12-3^T and *Bacillus alkalophilus* JCM 5262^T were used as reference strains for taxonomic characterization.

For phenotypic characterization, PYG medium (pH 10) containing 8 g peptone (Kyokuto), 3 g yeast extract (Kyokuto), 10 g d-glucose and 1 ml metal mixture [12] in distilled water containing 10 g Na_2CO_3 (pH 10) was used as the basal medium. A ten-fold concentrated alkaline solution (10% w/v NaHCO_3) was separately sterilized and then mixed with the autoclaved medium at a 10% ratio. The culture was incubated at 30°C for 72 h, and experiments were performed three times to confirm the reproducibility of results. Determinations of acid production from substrates using API 50CH (bioMérieux) were performed in at least duplicate at pH 10, as previously described [8]. Anaerobic conditions were produced by sealing mineral oil on top of the medium. The observations were performed until 2 weeks after the inoculations. Enzyme activity was tested using API ZYM (bioMérieux). Urease and nitrate reductase were tested using API 20 NE (bioMérieux). β-Galactosidase (ONPG) was tested using API 20 E (bioMérieux). Experiments on bacterial growth at pH 6–12 were performed as previously described [8]. The pH values of the spent medium after the cultivation of strain Bps-1^T changed from pH 8, 9, 10, 11 and 12 to pH 7.6, 8.9, 9.1, 7.9 and 8.3, respectively, after an incubation period of 6 days. The pH range for growth of strain Bps-1^T was pH 8–11, whereas the range for strains Bps-2 and Bps-3 was pH 8–12. The hydrolysis of macromolecules, and the oxidase and catalase tests were examined by the methods described in Cowan and Steel’s Manual [13] and in a report by Hirota *et al.* [8], by using 100 mM NaHCO_3/Na_2CO_3 buffer (pH 10). For the observation of negatively stained cells by transmission electron microscopy (TEM), cells were grown on PYG agar (containing 1.5% agar; pH 10) for 2 days. TEM sample preparation and observation were performed as previously described [14]. The cells were Gram-stain-positive and were facultatively anaerobic straight rods (0.5–0.7×1.1–1.5 µm) that use a pair of flagella (subpolar and centrally positioned) for motility (Fig. S1, available in the online Supplementary Material). Spherical endospores were formed in the terminal position and did not cause the sporangia to swell. The growth temperature ranges at pH 10 under aerobic conditions were examined using a temperature gradient incubator (Bio-Photorecorder, Type TN-2612, Advantec) by monitoring at OD_650 between 5–50°C. The growth temperature range for growth of strains Bps-1^T, Bps-2 and Bps-3 were determined to be 18–40, 15–40 and 18–40°C, respectively. The NaCl concentration range for growth of the isolates was determined to be 0–10% (w/v), with the optimum at 7% (w/v) by experimentally over the NaCl concentration range of 0–20%. (w/v). Physiological and biochemical characteristics are described in the species description. The isolates could be differentiated from phylogenetically neighbouring species of the genus *Bacillus* with validly published names on the basis of the characteristics listed in Table 1. Cells used for chemotaxonomic analyses and DNA–DNA relatedness of the isolates were harvested in the late logarithmic phase after aerobic reciprocal shaking (105 rpm) at 35°C in PYG broth containing 8 g peptone (Kyokuto), 3 g yeast extract (Kyokuto), 10 g d-glucose and 1 ml metal mixture [12] in distilled water containing 100 mM NaHCO_3/Na_2CO_3 buffer (pH 10). One mole of NaHCO_3/Na_2CO_3 buffer was separately sterilized and mixed with each autoclaved medium at a 10% ratio. Bacterial DNA was prepared by the method of Marmur [15]. The DNA base composition was determined as described previously [16], and indicated DNA G+C contents of strains Bps-1^T, Bps-2 and Bps-3 were 40.3, 40.7 and 40.3, respectively. Whole-cell fatty acid and isoprenoid quinones were analysed as described previously [17]. *meso*-DAP in the cell wall was identified by thin-layer chromatography (TLC) [18]. Lipo polysaccharides were analysed using the methods of Minnikin *et al.* [19] and Collins and Jones [20]. Major cellular fatty acids (>10%) of strain Bps-1^T were iso-C₁⁵:₀ (14.2%), anteiso-C₁⁵:₀ (50.2%) and C₁⁶:₀...
were negative for acid production from lactose and inulin. +, Positive; –, negative; W, weakly positive; ND, no data; v, variable; T, terminal; STC, subterminal.

The 16S rRNA genes of strains Bps-1

unidentified lipids (Fig. S2).

Table 1. Phenotypic and chemotaxonomic characteristics of Paralkalibacillus indicireducens gen. nov., sp. nov., Bps-1\(^T\), Bps-2 and Bps-3, and other related alkaliphilic Bacillus sp.

Taxa 1, Bps-1\(^T\); 2, Bps-2; 3, Bps-3; 4, Bacillus pseudofirmus DSM 8715\(^T\). Acid production from substrates was tested under anaerobic conditions in the present study. Acid production and growth characteristics data for species 4 were obtained using the same culturing and analytical methods as used for the novel isolates. Data on morphological characteristics and chemotaxonomic characteristics for species 4 is from Nielsen et al. [34] and Nogi et al. [35]. All the strains were positive for acid production from D-ribose, D-fructose, salicin, melibiose, sucrose and turanose. All the stains were negative for acid production from lactose and inulin. +, Positive; –, negative; W, weakly positive; ND, no data; v, variable; T, terminal; STC, subterminal to central; ST, subterminal.

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<td>Cell size (µm)</td>
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<td>0.5–1.1×1.1–2.1</td>
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<td>Temperature for range growth (°C)</td>
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<td>Optimum growth pH</td>
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<td>NaCl concn range for growth (% w/v)</td>
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<td>Optimum NaCl concn for growth (% w/v)</td>
<td>7</td>
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Acid produced from:

- Glycerol
- D-Arabinose
- D-Xylose
- D-Galactose
- D-Glucose
- D-Mannose
- Rhamnose
- Mannitol
- Methyl-α-D-glucopyranoside
- N-Acetyl-glucopyranoside
- Cellobiose
- Malto
- Trehalose
- Melezitose
- Raffinose
- Starch
- Glycogen
- Genitobiose
- Isoprenoid quinone
- DNA G+C mol% 40.3 %, 40.7 %, 40.3 %, 39.6 %

(14.0 %), with moderate amounts of (>5 % but<10 %) anteiso-C\(_{17:0}\) (8.5 %) found. A comparison of the fatty acid compositions of strains Bps-1\(^T\), Bps-2, Bps-3 and phylogenetically related strains is presented in Table S1. Menaquino-7 (MK-7) was detected by HPLC in the analysis of an extracted fraction from the cells. The cell-walls of these isolates contained meso-DAP. Polar lipids consisted of phosphatidylethanolamine, diphasphatidylglycerol, phosphatidylglycerol, one unidentified phospholipid and two unidentified lipids (Fig. S2).

The 16S rRNA genes of strains Bps-1\(^T\), Bps-2 and Bps-3 were PCR-amplified with the primers 9F (GAGTTTGATCCTGGCTCAG) and 1541R (AAGGAGGTGATCCAGGCC) [21]. The approximately 1.5 kb PCR product was sequenced directly by the dideoxynucleotide chain termination method using a DNA sequencer (ABI PRISM 3100; Applied Biosystems) with a BigDye Termination RR mix version 3.1 (Applied Biosystems), according to the manufacturer’s instructions. A BLAST search in NCIB [22] was used to identify the organism’s nearest phylogenetic relatives. The sequences were aligned, and the consensus sequence was determined by using the CLUSTAL W computer program [23]. A phylogenetic tree was reconstructed by using the maximum-likelihood [24], neighbour-joining [25] and minimum-evolution [26] methods in MEGA 6 [27]. The evolutionary distance matrix was calculated by using Kimura’s two-parameter model [28]. The similarity between sequences was calculated using the GENETYX computer program. The 16S rRNA gene sequences of strains Bps-1
(1439 bp), Bps-2 (1441 bp) and Bps-3 (1438 bp) were determined and compared with those of strains of species with validly published names in order to estimate sequence similarities. A maximum-likelihood phylogenetic tree of the three isolates together with their closely related neighbours was reconstructed (Fig. 1). The phylogenetic positions of the three isolates were depicted most clearly in the maximum-likelihood phylogenetic tree. The neighbour-joining tree was very similar to the minimum-evolutionary tree (Figs S3 and S4). Strain Bps-1 demonstrated exhibited sequence similarities of 96.1, 95.8 and 95.5 % with its closest relatives *Bacillus pseudofirmus* DSM 8715T, *Bacillus lindanensis* DSM 26864T and *Bacillus alcalophilus* DSM 485T, respectively. Strain Bps-1T was identical to strains Bps-2 and Bps-3, with 100 % 16S rRNA gene sequence similarity between all three strains.

On the basis of the results of 16S rRNA gene sequence analysis, although none of the reported species exhibited higher than 97 or 98.65 % similarity [29, 30], *Bacillus* no. AB042061; type species of the genus *Bacillus alcalophilus* was reconstructed (Fig. 1). The phylogenetic positions of the three isolates together with their closely related neighbours could be differentiated (Tables 1 and S1). However, *Bacillus* subtilis IAM 12118T (neutralophile and strictly aerobic characteristics [32]) and *Anaerobacillus arseniciselenatis* E1H1T (a strictly anaerobic strain [33]). However, there are several stains of species of the genus *Bacillus* that are similar to the novel isolates not only in terms of phylogenetic position, but also in terms of physiological characteristics. Therefore, we compared strain Bps-1T and the neighbouring alkaliphilic *Bacillus* spp., *Bacillus lindanensis* DSM 26864T and *Bacillus alcalophilus* JCM 5262T, instead of *Bacillus subtilis* IAM 12118T in Table 2. In addition, strain Bps-1T can be differentiated from all the strains listed in Table 2 including strains IEB3T, In2-9T and alkaliphilic *Bacillus* spp. on the basis of the flagella and spore morphology, chemotaxonomic characteristics, reduction of nitrate and growth characteristics (Tables 2 and S1).

**DESCRIPTION OF PARALKALIBACILLUS GEN. NOV.**

*Paralkalibacillus* (Par.al.kal.i.bacillus. Gr. prep. para, resembling; N.L. masc. n. *Alkalibacillus* a bacterial genus; N. L. masc. n. *Paralkalibacillus* resembling the genus *Alkalibacillus*).

Cells are Gram-stain-positive, facultatively anaerobic rods. Motile via a pair of flagella (subpolar and centrally positioned). Spherical endospores are produced at the terminal position in the sporangia. Endospores do not cause swollen sporangia. Catalase- and oxidase-positive. It grows in a medium containing 10 % (w/v) NaCl. Negative for the hydrolysis of gelatin, casein, starch, xylene, cellulose, and Tween 20, 40, 60 and 80. The quinone system is menaquione-7 (MK-7). The cell-wall peptidoglycan is based on meso-DAP. The major cellular fatty acids (>10 %) are anteiso-C15:0 and C16:0. Polar lipids comprise phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, one unidentified phospholipid and two unidentified lipids. Phylogenetically, the genus belongs to the family *Bacillaceae* in the class *Bacilli*.

The type species is *Paralkalibacillus indicireducens*.

**DESCRIPTION OF PARALKALIBACILLUS INDICIREDUCENS SP. NOV.**


Cells are straight rods (0.5–0.8 × 1.1–2.0 µm). Colonies are circular, slightly convex, creamy-white and 0.5–2.0 mm in diameter with entire margins on PYG-2 agar medium at 30 °C after 72 h cultivation. Urease and ONPG are positive. The cells are facultatively anaerobic and can reduce indigo carmine. The growth temperature range is 18–40 °C with the optimum at 33 °C (pH 10). The bacterium is halophilic with an NaCl concentration range for growth of 0–10 % (w/v), and the
optimum at 7\% (pH 10). Obligately alkaliphilic growth is at pH 8–11, with the optimum at pH 9–10 (30°C). Nitrate reduced neither nitrite nor N₂. Esterase (C₄), esterase (C₄)/lipase (C₈), leucine arylamidase, chymotrypsin, acid phosphatase, naphthyl-AS-BI-phosphorylase and β-galactosidase are positive, whereas alkaline phosphatase, lipase (C₁₄), valine arylamidase, cysteine arylamidase, trypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-
Table 2. Phenotypic and chemotaxonomic characteristics that differentiate Paralkalibacillus indicireducens gen., sp., nov., Bps-1T from species of related genera

| 1 | 2* | 3† | 4‡ | 5§ | 6|| |
|---|---|---|---|---|---|
| **Source** | Indigo fermentation liquor | Indigo fermentation liquor | Indigo fermentation liquor | Saline-alkali land | Faces | Sediment from soda lake |
| **Flagella** | Pair of subpolar and centre side | Peritrichous | Pair of subpolar | | | Non-motile |
| **Spore location** | T | STC | STC | C | TC | STC |
| **Spore shape** | Spherical | Ellipsoidal | Ellipsoidal | Ellipsoidal | Ellipsoidal | Ellipsoidal |
| **Swollen sporangia** | – | – | – | + | – | – |
| **Reduction of nitrate** | – | + | – | – | – | + |
| **Growth temperature range (°C)** | 18–40 | 10–45 | 20–40 | 5–45 | 15–40 | ND |
| **Optimum NaCl concn for growth (% w/v)** | 7 | 3 | 0 | 5–7 | | 6 |
| **Growth at 10 % NaCl (w/v)** | + | + | – | + | + | + |
| **Facultatively anaerobic** | + | + | + | + | | – |
| **Hydrolysis of:** | | | | | | |
| **Casein** | – | + | – | + | | ND |
| **Starch** | – | + | – | + | | ND |
| **Tween 60** | – | – | – | ND | | |
| **Menaquinone** | MK-7 | MK-6 | MK-7 | MK-7 | MK-6, MK-7 | ND |
| **DNA G+C mol%** | 40.3 % | 39.4 % | 39.0 % | 42.7 % | 36.5 % | 40.0 % |

*Data from Hirota et al. [9]
†Data from Hirota et al. [8].
‡Data from Dou et al. [36].
§Data from Nielsen et al. [34] and Nogi et al. [35].
||Data from Blum et al. [33].

β-glucosaminidase, α-mannosidase and α-fucosidase are negative. Acid is produced from D-ribose, methyl-β-D-xylopyranoside, D-fructose, N-acetyl-β-D-glucopyranoside, melibiose, sucrose, raffinose, turanose, D-lyxose, D-tagatose, 2-keto-glucuronate and 5-keto-glucuronate in anaerobic conditions. Acid is not produced from erythritol, D-arabinose, L-arabinose, D-xyllose, L-xyllose, D-galactose, D-glucose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl-α-D-glucopyranoside, amygdalin, arbutin, cellobiose, maltose, lactose, trehalose, inulin, starch, xylitol, genitobiose, D-tagatose, D-fucose, D-arabitol, L-arabitol and gluconate.

The type strain is Bps-1T (=JCM 31808T=NCIMB 15080T), which was isolated from Polygonum indigo (Polygonum tinctorum Lour.) fermentation liquor that was obtained from a craft centre in Date-City, Hokkaido, Japan. Additional strains of the species are strains Bps-2 and Bps-3. The genomic DNA G+C content of the type strain is 40.3 mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

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